# *IN VIVO* INFECTION BIOLOGY OF CONTAGIOUS BOVINE PLEUROPNEUMONIA

A Dissertation

by

TAMARA BROWNSEY GULL

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2007

Major Subject: Veterinary Microbiology

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Approved by:

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# ABSTRACT

*In vivo* Infection Biology of Contagious Bovine Pleuropneumonia.

(December 2007)

Tamara Brownsey Gull, B.A., University of Chicago; D.V.M., Tufts University School of Veterinary Medicine

Chair of Advisory Committee: Dr. L. Garry Adams

Contagious Bovine Pleuropneumonia (CBPP), caused by *Mycoplasma mycoides mycoides* small colony (MmmSC), is a devastating respiratory disease of cattle in Africa, Asia and the Middle East. Little investigation has been done on molecular disease pathogenesis and host response beyond soluble cytokine detection. This study developed and characterized models for three strains of MmmSC of varying severity. Strains used were Gladysdale, Ondangwa and Shawawa. Samples of bronchoalveolar lavage fluid, bronchial biopsy, nasal epithelial cells and blood were obtained prior to and at weekly time points post-infection. Microarray analysis of RNA extracted from samples revealed host cellular pathways and genes important in the pathogenesis of CBPP, including multiple immune system and inflammatory response pathways. A number of pathways whose influence on disease pathogenesis was not immediately clear were also activated, including pathways involved in amino acid synthesis, fat metabolism, and endocrine hormone responses. Microarray results were confirmed with real-time polymerase chain reaction (RT-PCR) of selected genes. Comparative RT-PCR analysis of selected genes

between the three strains of MmmSC revealed genes possibly responsible for differential strain virulence, including interleukins 1B, 6, 8, and 18 and the gene nuclear factor of kappa light polypeptide gene enhancer in B cells inhibitor, alpha (NFKBIA). A similar analysis of selected genes between survivors and nonsurvivors of the virulent Gladysdale strain of MmmSC suggested genes involved in survival, including interleukin 8, calmodulin 2 (CALM2), and NFKBIA. Avenues of additional study were identified.

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# CHAPTER I

# INTRODUCTION

## BACKGROUND

Contagious Bovine Pleuropneumonia (CBPP) is a pulmonary disease of cattle and related large ruminants caused by the bacterium *Mycoplasma mycoides mycoides* small colony (MmmSC). CBPP is the only bacterial disease on the List A of pathogens of concern to the World Organization of Animal Health, the Office International des Epizooties (OIE) (http://www.oie.int/eng/maladies/en_classification.htm#ListeA). It is also a United States Department of Agriculture (USDA) high consequence livestock pathogen. CBPP has profound economic effects on the cattle industry in many parts of Africa and coastal Europe due to overt losses and sanctions against exportation of cattle[1], and it is estimated that losses in Africa alone exceed $2 billion per year[2]. Public health concerns are also associated with CBPP; the incidence of childhood malnutrition increases in areas experiencing large-scale cattle slaughter due to CBPP[3]. The disease is undergoing resurgence in parts of Africa[1], and recent outbreaks have also occurred in several locations in southern Europe. Due to its long absence from the Western hemisphere and the intensive high-stocking-density methods of dairy and feedlot cattle production, reintroduction of CBPP to the United States (whether accidental or

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deliberate) could have devastating effects on the cattle industry.

**THE DISEASE**

Contagious Bovine Pleuropneumonia has been clinically described in cattle populations in manuscripts dating as far back as 1550[4]. It was first believed to be a "paralysis of the lung" and was not recognized as a contagious disease until 1794[5]. In the mid-1800s Louis Willems demonstrated that the disease could be induced via inoculation of naïve animals with pulmonary exudates from infected animals. Control efforts in the United States were implemented prior to the identification of the causative organism and the disease was eradicated from the US by 1892. The eradication of CBPP was the driving force behind the establishment of the Bureau of Animal Industry, the precursor to today's United States Department of Agriculture. The microbe responsible for CBPP was identified by Nocard and Roux in 1898[6], and shortly thereafter Dujardin-Beaumetz recognized the small size of the organism and showed how it could be separated from larger bacteria through filtration[7].

Cattle are the primary susceptible species for CBPP, but reports exist of affected water buffalo, yak, bison and reindeer[4]. Small ruminants such as goats and sheep have been shown to harbor the mycoplasma but have not been shown to develop disease, and their role in the spread of CBPP is unclear[8]. There may be differential susceptibilities of different breeds or species of cattle, but this remains unconfirmed[9]. The causative organism is transmitted by direct contact.

Clinical signs in affected animals include tachypnea, fever, cough, respiratory distress, lethargy, inappetence, weight loss and pleurodynia. Decreases in or cessation of milk production may occur in dairy animals. Lameness and increased recumbency are seen in younger animals with joint involvement. Direct economic losses occur from death, weight loss, decreased milk yield, decreased working ability and decreased fertility. Indirect effects include an increase in human malnutrition in affected countries, particularly those imposing slaughter control measures[3]. Although the disease is characterized by respiratory signs, animals develop mycoplasmemia and demonstrate systemic effects. At necropsy, animals with CBPP demonstrate often-unilateral serofibrinous pleuropneumonia characterized by severe interstitial edema and fibrosis[4,10]. Large quantities of pleural fluid may be found in acute cases, and fibrinous adhesions between pulmonary visceral and parietal pleura are common. Pleura are thickened with layered fibrin deposits frequently described as omelette-like. The lungs are often reported to have a marbled appearance due to thickening of interlobular septae and color changes. Necrotic areas of lung may develop, and these areas may become sequestered via formation of a thick layer of fibrous tissue. These sequestra may persist for months or years after an affected animal has apparently recovered from the disease. Thoracic lymph nodes in animals with CBPP may be enlarged and edematous and may demonstrate necrotic areas; mycoplasma can be isolated from the lymph nodes[11]. Septic arthritis, with joint distention, synovitis and cartilaginous erosions, may be seen in younger animals (usually <6 months of age). Renal infarcts have been reported in all age groups[12]. Exudative pericarditis and peritonitis have also been reported. The causative

organism has been isolated from lymph, serum, plasma, urine and semen, further demonstrating the systemic nature of the disease[11-16].

The incubation period of naturally-occurring disease varies between 5-207 days, but is normally between 31-45 days[17]. There are distinct differences in morbidity and mortality between the African and European strain clusters of MmmSC; some African strains may demonstrate up to 90% morbidity and up to 70% mortality, while European strains often show only up to 30% morbidity and <10% mortality. Disease may be hyperacute, with severe pleuropneumonia and death in 1-10 days; acute with cough, fever, pneumonia and pleurodynia lasting ten days to several weeks; subacute with intermittent fever, anorexia and mild pneumonia; and chronic. Cattle may shed the organism for weeks prior to demonstrating illness, and prior to seroconversion. The chronic form of CBPP may occur upon initial infection or following either acute or subacute disease and results in the development of encapsulated infected sequestra within pulmonary tissue. Abattoir studies have shown that up to 80% of animals "recovered" from CBPP had sequestra at slaughter[17]. Sequestra are believed to be one of the methods by which CBPP is maintained in cattle populations, as animals with sequestrae may periodically shed MmmSC[18]. Intermittent shedding may continue for up to two years. So while subacutely or acutely-infected animals may only appear clinically affected for weeks, the actual duration of infection with MmmSC may be considerably longer[4]. The dynamics of CBPP infection have been mathematically modeled in both sedentary[19] and nomadic[20,21] cattle populations in Africa using incidence data, and both models support the presence of carrier animals in maintaining the disease within their respective

populations. The factors governing recrudescence of mycoplasma shedding are undetermined; an experimental attempt to reactivate a sequestered infection was unsuccessful[22].

## INCIDENCE AND PREVALENCE

CBPP has primarily been a problem in Africa, particularly the sub-Saharan and central regions. It had been on the decline in the late 1960s and 1970s due to widespread vaccination programs in many affected countries. However, the decrease in government-sponsored vaccination programs since the 1980s has allowed the disease to spread virtually unchecked in many areas. Several countries previously thought free of the disease have experienced recurrences in the last two decades[1], and reports indicate that these new outbreaks are of significantly higher morbidity[23]. If anything, CBPP incidence is underreported in Africa due to inadequate surveillance. The disease remains of vital importance to the African cattle industry and, with the increasing control of rinderpest, has become the major infectious disease of livestock on the African continent (**Figure 1**).

While Europe has yet to experience any CBPP outbreaks in the 21st century, several outbreaks occurred in the 1990s in Portugal, Spain and Italy. Europe has experienced outbreaks at least once per decade despite vigorous eradication efforts[24]. The Middle East has had outbreaks in Saudi Arabia, Pakistan, Kuwait and Qatar in the past decade, and Southeast Asia has had outbreaks in Bangladesh and Myanmar. Sporadic outbreaks have been seen in China and possibly Mongolia as well. However, many Asian countries have not reported CBPP status to the OIE, so actual incidence

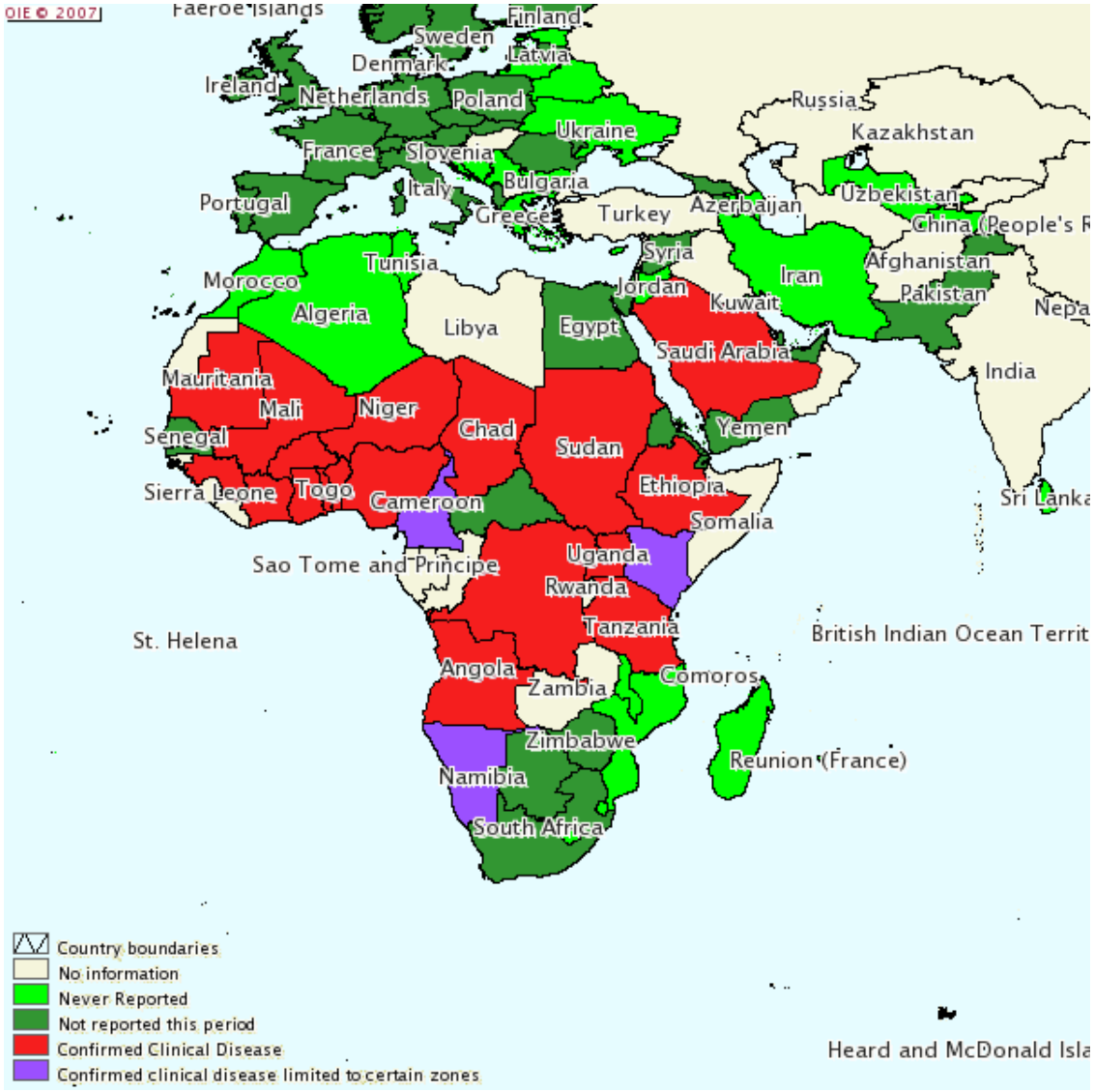



**Figure 1. Reported Occurrence of Contagious Bovine Pleuropneumonia in Africa.**

January through June 2005[219].

may again be underreported.

CBPP was eradicated from the United States in 1892 and from Australia in 1973. Eradication in both the U.S. and Australia was dependent on strict control of cattle movement, herd-scale slaughter and financial remuneration to owners. Unfortunately such methods are not feasible in undeveloped nations, as they lack the governmental and civil resources to effectively restrict movement and the financial resources to compensate animal owners. Additionally, cultural issues involving traditional nomadic lifestyles and migration patterns and the social status value of cattle often make animal owners unwilling to comply with such movement control and eradication measures. As no viable treatment for CBPP exists, vaccination is the control method of choice at this time. However, there are difficulties with this method that will be discussed.

**PREVENTION AND TREATMENT**

Vaccination strategies have been employed against CBPP since at least the latter half of the 19th century. Early vaccination measures involved inoculating pleural fluid from infected animals into naïve animals. This method of vaccination resulted in the death of 1-10% of vaccinates[25]. Since then, vaccination against CBPP has remained a struggle[26]. Multiple vaccine formulations have been developed but all have shown drawbacks including the induction of undesirable side effects and lack of consistent protective or long-lived immunity[25]. Over ten inactivated vaccines have all failed to provide adequate protection, while occasionally resulting in sensitization of inoculated animals to the organism. Attenuated (by serial subculture) vaccines have frequently

resulted in variable protection, severe local reactions, clinical disease and occasionally death of vaccinated animals[26-28]. The currently in-use T1/44 attenuated vaccine requires annual revaccination for protection, which is economically and logistically impractical in many African countries[1]. It has been reported to cause clinical disease[29], and requires careful handling to maintain efficacy. It is, advantageously, specifically identifiable via PCR[30]. Investigation is ongoing toward specific-component vaccines for CBPP[31], but efforts are hampered by the expense and logistical difficulty of conducting field trials. Genetically-modified vaccine strains of MmmSC have not been attempted. Development of new CBPP vaccines is unlikely given the political, economic and logistic challenges present in endemic areas[2,32].

No effective antibiotic therapy is available for CBPP. While antibiotic therapy has been shown to resolve the clinical signs of CBPP, lesions were still present at necropsy in treated animals[33,34]. Fluoroquinolone antibiotic treatment has been shown to reduce the spread of the disease to in-contact susceptible animals, but the treated animals maintained the characteristic necropsy lesions[35]. It has been suggested that treatment of CBPP-infected animals with antibiotics or anti-inflammatory drugs may contribute to the development of the chronic carrier state and sequestra formation, possibly since the majority of antibiotics used based on *in vitro* sensitivities are bacteriostatic[36]. No studies have specifically investigated this hypothesis. Treatment of CBPP-infected animals with antibiotics is actively discouraged by the OIE.

## THE ORGANISM

MmmSC is in the class *Mollicutes*, order *Mycoplasmatales* and family *Mycoplasmataceae*. Like all mycoplasmas, it is a wall-less prokaryote with a low G+C content (24%) and is among the simplest self-replicating organisms known[37]. MmmSC is a member of the *M. mycoides* cluster, a closely-related group of mycoplasmas that cause disease in ruminant species (**Table 1**). The classical *M. mycoides* cluster contains six organisms that share many biochemical and genetic determinants, and differentiation between the organisms can be difficult[10,38]. Interestingly, the bacteria in the *M. mycoides* cluster appear to be more closely related to organisms in genii *Entomoplasma*, *Mesoplasma* and *Spiroplasma* than to other organisms in genus *Mycoplasma*[39,40]. MmmSC, like most mycoplasmas, is highly host-specific in its ability to cause disease. There are more than 60 known strains of MmmSC divided into two clusters, African/Australian and European. The full genome sequence (1.2 Mbp) has been published only for the MmmSC type strain PG1[41], but the genome for the African strain Shawawa has also been sequenced [S.J. Geary, personal communication]. Initial comparison of the two genomes has revealed minimal differences. Although the type strain is known to be pathogenic, it fails to fall into either of the two major geographic clusters and its origin is unknown[42].

The African/Australian and European clusters of MmmSC are primarily differentiated by an 8.84kb deletion in the genome of European strains and an associated decrease in virulence, with the African strains demonstrating significantly higher

**Table 1. Diseases caused by mycoplasmas of the classical *M. mycoides* cluster.** Table adapted from Persson, 2002.

| **Species** | **Reference Strain** | **Main host species (secondary species)** | **Disease(s)** |
|---|---|---|---|
| *M. capricolum capricolum* | California kid [T] | Goats, sheep (cattle) | Arthritis, mastitis, pneumonia |
| *M. capricolum capripneumoniae* | F38 [T] | Goats (sheep) | Pleuropneumonia (CCPP) |
| *M. mycoides capri* | PG3 [T] | Goats | Arthritis, pleuropneumonia |
| *M. mycoides mycoides* LC | Y-goat [R] | Goats, sheep (cattle) | Arthritis, peritonitis, septicemia, pleuropneumonia |
| *M. mycoides mycoides* SC | PG1 [T] | Cattle (buffalo, goats, sheep) | Pleuropneumonia (CBPP), arthritis |
| *Mycoplasma* bovine group 7 | PG50 [R] | Cattle (goats, sheep) | Arthritis, mastitis, calf pneumonia |

[T] Type strain
[R] Reference strain

virulence[43]. There has been intense interest in investigating virulence determinants of MmmSC and other mycoplasmas, since they are not known to secrete toxins or to express specific adherence factors, yet demonstrate dramatic variations in disease severity. Their minimal genome also suggests that so-called virulence factors likely are also integral to the normal metabolism of the mycoplasma. Sequencing of the deletion area present in the African strains has been conducted and has tentatively identified five open reading frames (ORFs) including a membrane lipoprotein and a putative abc transporter protein[43]. No research has been published investigating the virulence of single deletion mutants of these five ORFs. Two of these five ORFs have been identified as *gtsB* and *gtsC*, which are involved in glycerol transport. Hydrogen peroxide is an end-product of glycerol metabolism, and strains naturally lacking these genes produce less hydrogen peroxide *in vitro*[44]. This difference in hydrogen peroxide production has been hypothesized to be a virulence factor differentiating the African and European strains of MmmSC[45]. The membrane lipoprotein encoded in the deletion area, LppB, is present in several other bacteria in the *M. mycoides* cluster[43,46]. It has also been proposed as a virulence factor simply because of its probable surface membrane location, where it might interact with host cells.

MmmSC is known to undergo high-frequency surface antigen variation, which complicates reliable serologic detection of the organism[47,48]. This phase variation of surface lipoproteins has been proposed as a virulence factor[47], and several other lipoproteins whose impact on virulence is unknown have been identified as antigenic epitopes[43,49-52]. Investigation has revealed at least eight immunogenic MmmSC epitopes

against which an infected host develops a serum antibody response, and six of these are known to be lipoproteins[53], which supports the possible role of lipoproteins as virulence factors. Additionally, the galactan capsule of MmmSC may be a virulence determinant. Galactan is known to promote microbial binding to host cell surfaces and to provide resistance to phagocytosis. Its similarity to bovine pneumogalactan, produced by respiratory epithelial cells, may induce the formation of autoantibodies. Finally, injection of capsular galactan is also known to induce toxic effects on the host[54,55].

Another potential virulence determinant suggested in recent research is a single nucleotide polymorphism (SNP) within the gene for 6-phospho-β-glucosidase (*bgl*). Strains of MmmSC with the isoform Bgl $Val_{204}$ demonstrate higher pathogenicity than those with Bgl isoform $Ala_{204}$[56]. The proposed mechanism is that strains with the $Val_{204}$ isoform have decreased hydrolyzing activity and may be better equipped to survive in environments containing high levels of β-D-glucosides such as the bovine lung. Investigation of virulence factors of mycoplasmas is complicated by the difficulty of genetically modifying these organisms. Mycoplasmas are remarkably resistant to classical methods of mutagenesis, a characteristic that has frustrated researchers for decades. As examples, transformation has only been possible in mycoides cluster organisms in the presence of high concentrations of polyethylene glycol, and then at very low success rates compared to many other bacterial species[57]. Additionally, only two transposons, Tn*916* and Tn*4001*, have been successfully applied in mycoplasmas[58,59], although these have not been applied to MmmSC. However, the generation of artificial *oriC* plasmids which have successfully been used to transform MmmSC and the related

organism *Mycoplasma mycoides mycoides* large colony (MmmLC)[60] may herald incipient advances in the manipulation of the mycoides cluster genome. Unfortunately, it appears that plasmids used for transformation may need to be individually designed for the target mycoplasma species, as these artificial plasmids exhibit often stringent host specificity[60].

Mycoplasmas are often characterized by the presence of insertion sequences coding for putative transposases in their genomes. MmmSC is known to contain three such insertion sequences: ISMymy1[61]; IS1634[62]; and IS1296[63]. Approximately eight copies of ISMymy1 occur in all tested strains of MmmSC[61], and this sequence is also found in some strains of *Mycoplasma bovis*. Roughly 60 copies of IS1634 occur in all strains of MmmSC[62], but no other mycoplasmas. Variable numbers of copies of IS1296 occur, and this IS may be used to differentiate European versus African cluster strains of MmmSC[63]. The presence of these putative transposases in the mycoplasma genome is difficult to explain given the resistance of the mycoplasmas to deliberate transposon insertion and the apparent stability of a genome that includes what appear to be multiple ancestral transposons.

## HOST RESPONSE TO INFECTION

The mechanisms by which MmmSC evades or circumvents the host immune response are poorly understood, as are the details of how the host responds to infection. *In vitro* studies have demonstrated that pathogenic strains of MmmSC induce TNFα production by alveolar macrophages but fail to induce procoagulant activity or nitric

oxide formation[64]. An *ex vivo* experiment generated evidence that IFNγ-producing $CD4^+$ T cells were necessary for recovery from CBPP, but this study did not differentiate between recovered animals with necropsy lesions (chronically-infected animals) and those without (animals in which evidence of disease was no longer present)[65]. A follow-on study suggested that live MmmSC could secrete a substance that induced apoptosis in both lymphocytes and granulocytes[66]. Although this substance has not been further defined, the study authors suggest that hydrogen peroxide should be considered for the role. *In vivo* studies of the immune response have demonstrated that cell-mediated immunity is important in the resolution of clinical signs[65]. Additionally, MmmSC appears to be able to inhibit concanavalin A-induced blastogenesis and IFNγ production by PBMC and lymphocytes[67], further intimating that cell-mediated immunity may be necessary for control of infection. Contrary to these observations is one study that showed that passive immunity to CBPP could be transferred via serum[68]. Investigation of the humoral immune response to infection has shown a potent IgA response local to infection and a weaker systemic IgG response[53], and the response is stronger in animals infected with more virulent strains of MmmSC. A different study suggested that increased IgA production was correlated with less severe disease, while other immunoglobulin isotypes were similar between animals with acute disease and those with subacute or chronic disease[69]. Multiple studies have suggested that the magnitude of the host response is in part responsible for the severity of lesions observed in CBPP, but the extent of this contribution is undetermined[31,70,71].

## ANIMAL MODELS OF DISEASE, DETECTION AND VACCINE STUDIES

Most *in vivo* studies of CBPP have used cattle, as it has proven extremely difficult to cause disease in other ruminant species and transmission in other ruminant species does not appear to occur as readily as in cattle. A few mouse models have been used, particularly in vaccine studies, but mice fail to develop the characteristic lesions of CBPP and researchers instead rely on the induction of mycoplasmemia to evaluate response[27,72-77]. Multiple bovine models have been used for infection, including direct contact, blind endotracheal or endobronchial inoculation and nasal inoculation[9,22,53,65,69]. Previous models inoculated various growth media (agar, Hayflick medium, etc.) in addition to the bacterial isolate, and bacterial numbers were variable[22,65,78]. Inocula could not be quantified in models of direct contact infection. Species, breed or type of cattle were frequently not reported, and some studies of natural infection did not report strain type of the organism beyond 'naturally-occurring field strain'. Dose dependence has been cited as a factor in both disease induction and vaccine efficacy[26,27,34,74,79].

*In vitro* studies have used fetal bovine nasal epithelial cell cultures, bronchial epithelial cultures, or bovine macrophages or lymphocytes[45,64,66]. None of these systems replicates the complex environment found in the lung. Cultured cell lines, while providing the target cell type, fall short of replicating the animal environment. Epithelial cells are polarized *in vivo*, and each cell surface has very different roles, surface molecules, and capabilities. Polarized epithelial cell models are difficult to construct and maintain, and none of the MmmSC studies have made use of them. Single cell type

models also eliminate the complex interactions seen between cells in the animal host, such as the cellular trafficking between epithelial cells and the subepithelial cell layers, endothelial cells, white blood cells and the cells of the immune system. Even the proportions of type 1 and type 2 respiratory epithelial cells in a culture system may affect the results of an *in vitro* experiment by altering the amount of secreted substances such as mucus and surfactant. While easier to manipulate than the whole animal model, the drawbacks of *in vitro* models may present difficulties in interpreting results.

No studies have investigated the host global immune response to infection with MmmSC; all have focused on one arm of the immune system. The lack of confluence of models makes comparisons of their conclusions difficult. There is also a lack of studies investigating differences during the course of infection in recovered animals with and without lesions at necropsy. Observers of CBPP outbreaks since 1873[25] have acknowledged the existence of a 'resistant' subgroup of animals that never develop lesions or disease, but little has been done to investigate this phenomenon. One preliminary study investigating genetic markers of susceptibility identified the BoLA allele cbb (DRB3*2101) as being predominantly associated with resistance to CBPP and the lba allele being associated with susceptibility, although study size precluded significance[25].

Diagnosis of infection is complicated. Sample source has a large impact: blood is easy to obtain but less reliable, while bronchial secretions are more reliable but more labor-intensive to acquire. Nasal secretions are unreliable sample sources, and urine has only been minimally investigated[16,80-82]. The current "gold standard" herd-level blood

test per the OIE is the complement fixation test (CFT). This test is extremely specific during acute infection, but is less useful in animals during both early and chronic infection[83]. In addition, the CFT is complex and requires stringent controls and technical training; it has proven difficult to adapt to a pen-side format. Some chronically-infected animals fail to seroconvert on the CFT despite intermittently shedding the organism (T. Gorton, unpublished observations). Even animals with acute disease typically do not seroconvert until after the onset of clinical signs. Passive hemagglutination tests may help identify early infections through detection of the IgM response, but they lack sensitivity and specificity[18,84]. A latex agglutination test has been developed that demonstrates sensitivity similar to CFT but is much simpler to perform; however, it suffers the same drawbacks as CFT in early or chronic infection[85,86]. Culture of the organism is time-consuming due to slow growth and the requirement for selective media; even after culture, specific biochemical tests are required to definitively identify the organism. Postmortem use of immunohistochemistry has proven useful where culture techniques are inappropriate[11], but still suffers cross-reactivity with other mycoides cluster organisms. Other serologic tests have been plagued by cross-reactivity of antibodies within the *M. mycoides* cluster of organisms, resulting in low sensitivity and specificity of any single serologic test[87]. Other methods of detection have been proposed for MmmSC. Both ELISA and immunoblot assays suffer from serologic cross-reactivity, and are not amenable to field use[88,89]. A number of PCR-based tests have been developed that are sensitive and specific, but require restriction endonuclease digestion and gel electrophoresis after PCR for results to be useful[38,80,90-94]. None of these PCR-based tests

have proven adaptable to field conditions. A real-time PCR test was recently shown to be sensitive and specific, and required no special processing of a sample of bronchial secretions[95]. This test was developed for use in a portable platform, but no field trials have been conducted. A sensitive, specific and field-adaptable test for MmmSC, using an easily-obtainable bovine sample, has so far remained elusive.

**AIMS**

The overall aim of this study is to investigate the temporal global host response to infection with MmmSC at the transcriptional level, in both acute and chronic models of infection. The second chapter of this dissertation discusses the development of the models, timelines, clinical findings and morphological findings. Both acute and chronic models will be described from infection through chronicity. The third chapter will investigate the temporal transcriptional response of the bovine host in several tissue types. This discussion will focus on the identification, through dynamic Bayesian modeling analysis, of specific mechanistic genes and pathways that appear relevant during the course of infection. The fourth chapter will describe selected genes in the contexts of differential strain severity and survival versus nonsurvival. Cell types investigated will include macrophages and neutrophils obtained via bronchoalveolar lavage, bronchial epithelial cells obtained via bronchial biopsies, nasal epithelial cells and blood. Selected genes and cellular pathways will be discussed in greater depth in the context of published knowledge on MmmSC and Contagious Bovine Pleuropneumonia.

# CHAPTER II

# ACUTE AND CHRONIC MODELS OF CONTAGIOUS BOVINE PLEUROPNEUMONIA

## INTRODUCTION

Previous studies involving bovine models of CBPP have used a variety of isolates, inoculation methods and bacterial numbers. Due to the difficulty of establishing a viable *in vitro* model of CBPP, *in vivo* studies are necessary in order to further determine the effects of infection on the bovine host. While direct contact with cattle already shedding the organism is the natural method by which naïve animals are infected[37], this manner of infection can be unreliable, does not permit quantitation of the infective dose, and results in prolonged incubation periods before animals demonstrate disease. With a direct-contact model of infection it is impossible to determine when, or even if, a particular susceptible animal received an infective dose of organism. Dose dependence has been cited as a factor in both disease induction and vaccine efficacy[26,27,34,74,79]. Inoculation of cattle by other routes (nasal or endobronchial) allows accurate determination of both quantity of organism and incubation period. Infection of cattle with field strains of MmmSC exhibiting differing virulence under the same experimental protocol should permit better comparison of the effects of these strains on the bovine host. Side-by-side evaluation of these acute and chronic models of CBPP infection using identical techniques may allow more relevant comparisons to be made

between the models and could lead to the discovery of subtle differences in both disease pathogenesis and host immune response.

The goal of this study was to infect separate groups of cattle with identical numbers of three different strains of MmmSC and observe their clinical responses, death versus recovery, and pathologic lesions at necropsy. This study produced both acute and chronic disease models which may be directly compared. In addition, sampling of bronchoalveolar lavage fluid, nasal epithelial cells, blood and bronchial epithelial cells was done at multiple time points following infection; samples obtained were used in other studies of CBPP. Side-by-side evaluation of acute and chronic models of CBPP infection using identical techniques should allow more relevant comparisons to be made between the models and may suggest avenues of exploration in both disease pathogenesis and host immune response.

## MATERIALS AND METHODS

**Experimental animals.** All experimental protocols were approved by the USDA/DHS Plum Island Foreign Animal Disease Center (PIADC) Animal Care and Use Committee. Mixed-breed *Bos taurus* cattle between 6-8 months of age were obtained from a commercial source commonly used by the USDA Agricultural Research Service. Each animal was individually identified by a numbered ear tag. Animals received an initial physical evaluation and anthelmintic treatment with ivermectin (Ivomec injectable 1% solution, 200 µg/kg SQ, Merial, Duluth GA) before being used for the study. Animals were acclimated to the experimental rooms for three weeks prior to the start of the

experiment. They were housed in groups of five in separate rooms of the indoor biocontainment facility at the USDA/DHS Plum Island Foreign Animal Disease Center. Alfalfa pellets and fresh water were provided *ad libitum*. Pens were cleaned daily. All animals were observed by experienced husbandry staff at least once daily, and rectal temperatures were taken weekly prior to inoculation and daily thereafter. Temperatures, clinical signs of illness and attitude were recorded on daily observation sheets.

**Sample collection.** At the start of each sampling procedure, individual animals were restrained in a squeeze chute and headgate integral to the room. A rope halter was applied and used for further restraint as needed. Two 10ml blood samples were obtained via jugular venipuncture. One blood sample was aliquoted into Tri-Reagent BD (Molecular Research Center, Cincinnati OH) at a 1:5 ratio for RNA isolation. The other was allowed to clot and be centrifuged for serum. Nasal swabs for culture and PCR were obtained using a sterile guarded rayon uterine culture swab (Jorgensen, Loveland CO). The guarded swab was inserted up the left nostril 10-15cm, the swab extended and the mucosa gently rubbed. The swab was retracted into its guard before withdrawal. A sample of nasal mucosal cells was obtained with a sterile guarded uterine cytology brush (Jorgensen). The guarded brush was inserted up the left nostril to the level of the medial canthus of the eye, the sampling brush was extended and the mucosa swabbed 5-10 times. The brush was retracted into its guard prior to withdrawal. The brush assembly was amputated into a 2ml microcentrifuge tube containing RNAlater (Ambion, Austin TX) and stored at 4$^{o}$C until further processing. Prior to inoculation with MmmSC, all cattle underwent endoscopic bronchalveolar lavage (BAL) using a 1.6m videocolonoscope

(Olympus, Center Valley PA). The endoscope was introduced through the right nostril and visually guided through the larynx and into the right caudal lung lobe until bronchial size prevented further advancement. Two aliquots of 60ml sterile physiologic saline (Abbott, Abbott Park IL) were instilled through a sterile single-use double-guarded aspiration catheter (Mila International, Erlanger KY) threaded through the biopsy channel of the endoscope. Each aliquot was instilled and reaspirated into the delivery syringe. One 2ml aliquot of recovered fluid was placed on ice for culture and PCR of MmmSC. The remainder of the aspirated BAL fluid was aliquoted into 2ml microcentrifuge tubes and centrifuged at 10,000 x *g* at room temperature for 10 minutes to pellet the cells. The supernatant was discarded and cells were resuspended in 2ml RNAlater (Ambion). Resuspended cells were stored at 4$^{o}$C until further processing. Following the BAL, the endoscope was withdrawn until a bronchial bifurcation could be visualized. Five milliliters of 2% lidocaine (AstraZeneca, Wilmington DE) was instilled through the biopsy channel. A 2.5mm biopsy forcep (Precision Endoscopy, Hunt Valley MD) was passed down the biopsy channel. Two bronchial mucosal biopsies were obtained and placed in RNAlater. Biopsy specimens were stored at 4$^{o}$C until further processing. Following the biopsy procedure, the endoscope was withdrawn completely. The endoscope was disinfected and the biopsy channel flushed thrice with 70% isopropanol and rinsed with sterile water prior to use on the next animal. Following the sampling of all cattle in a treatment room the endoscope was disinfected as described above, then sterilized in glutaraldehyde solution (Cidex, Johnson & Johnson, New Brunswick NJ) per the manufacturer's instructions. Sterilized endoscopes were rinsed with sterile water and

stored in the anteroom of the animal housing room for the next sampling day. The endoscope was not transferred between animal rooms; a separate scope was used in each room.

**MmmSC strains.** MmmSC Australian strain Gladysdale (Animal and Plant Health Inspection Service, USDA, Plum Island Animal Disease Center, Greenport NY) was used for the acute infection model and African field strains Ondangwa and Shawawa (Dr. Leon Prozesky, University of Pretoria, Onderstepoort, South Africa) were used for the chronic models. Strains were grown in Fortified Commercial medium (Difco, Franklin Lakes NJ) at 37$^{o}$C for 72 hours and were centrifuged for 15 minutes at 12,000 x *g* and 4$^{o}$C, then resuspended in phosphate buffered saline prior to inoculation.

**Pulmonary infection.** One day after the preinoculation sampling, $10^{10}$ MmmSC suspended in 2ml phosphate buffered saline were inoculated into the right caudal lung lobe via an aspiration catheter threaded through the endoscope biopsy channel. The catheter was flushed with 10ml PBS and the endoscope withdrawn. Sampling as described above was conducted at Day 1 post-infection and weekly until the conclusion of the experiment.

**Evaluation of clinical disease.** Animals were observed daily by animal husbandry staff familiar with clinical signs of CBPP and weekly by a board-certified large animal internist and a board-certified pathologist. Rectal temperatures and clinical observations were recorded on daily observation sheets.

**Necropsy procedure.** Cattle were euthanized with a pentobarbital overdose administered intravenously (Sleepaway, 26mg/lb, Fort Dodge Animal Health, Fort Dodge

IA) and necropsied at the time that clinical signs of disease suggested that death was imminent. If clinical disease was inapparent or observed but resolved, animals were euthanized and necropsied between Days 36-43 post-infection depending on necropsy room and animal handler availability. Euthanized animals were immediately necropsied by a board-certified pathologist assisted by a board-certified large animal internist. A postmortem bronchoalveolar lavage was done using one 60ml aliquot of physiologic saline and an aspiration catheter after the lungs were removed from the thoracic cavity, and samples were handled as described above. All organs were examined for gross pathology. Samples of lung, tracheobronchial lymph node and joint, as well as any other grossly abnormal tissues, were fixed in 10% buffered formalin for histopathology. Tissue samples of lung, bronchial mucosa, tracheobronchial lymph node and kidney were minced and placed into a 2ml microcentrifuge tube in RNAlater, then stored at 4$^{o}$C until further processing. Carcasses were incinerated in accordance with PIADC protocols.

**Culture of MmmSC.** Aliquots of BAL fluid and nasal swabs were plated or swabbed onto Fortified Commercial agar (Difco) and grown at 37$^{o}$C for a minimum of 72 hours. Subculture was performed if necessary. Colony morphology was evaluated under a dissecting microscope. Representative colonies were selected for evaluation by PCR.

**PCR of MmmSC from BAL fluid.** One milliliter aliquots of BAL fluid were centrifuged at 12,000 x *g* for 15 minutes at 4$^{o}$C. The supernatant was removed and the pellet resuspended in 500 µL phosphate buffered saline. The samples were boiled for 10 minutes. Next, 1 µL of the sample was combined with 0.9 µM concentration of each forward and reverse primer, 0.1 µM concentration of probe and TaqMan Universal PCR

Master Mix (Applied Biosystems) in a total volume of 20 µL. Real-time PCR was performed with this reaction mixture on the SmartCycler I (Cepheid, Sunnyvale CA). Reaction conditions included 2 minutes at 50°C followed by 10 minutes at 95°C and then 40 cycles of 15 seconds at 94°C and 60 seconds at 60°C. The default threshold limit was set at 30 and the optics detection was turned on for the cycle steps at 60°C. Data were exported to Microsoft Excel for analysis. Primers used were sequence 5'-ATGGACGAAAGTCTGATGAAGCAATGC-3' and 5'-TCTGGTAAGGTACTGTCAAGATAAAGTCAT-3', and the probe used was sequence 5'-6FAM-ACAACAGAGATTTACAAC-MGBNFQ-3', in which 6FAM is the fluorescent dye 6-carboxyfluorescein and MGBNFQ is a minor groove binding non-fluorescent quencher. This probe targets a unique polymorphism within the MmmSC 16S RNA gene *rrnA*[95].

**Histopathology.** Tissues collected at necropsy and fixed in 10% buffered formalin were embedded in paraffin and sectioned at 5 µm onto glass slides for light microscopy. Slides were stained with hematoxylin and eosin and scored by a board-certified pathologist familiar with CBPP lesions and blinded as to with which strain the steer had been infected.

**Electron microscopy.** Bronchial epithelial tissues collected at sampling time points via biopsy forceps and at necropsy were processed for thin section analysis. Small pieces of bovine lung containing characteristic lesions were fixed with a solution containing 10% neutral buffered formalin. After 1-5 days, necropsy tissues were trimmed into 3 mm x 10 mm strips and fixed with a solution containing 2.5%

glutaraldehyde in 0.1M cacodylate buffer (pH 7.4) for 24 hrs. Biopsy samples were processed similarly, but were not trimmed. After rinsing with 0.1M cacodylate with 10% sucrose, the tissue was post-fixed with 1% osmium tetroxide and 1.5% potassium ferricyanide for 1 hr at 4$^{o}$C. The tissues were stained *en bloc* with 2% aqueous uranyl acetate, dehydrated with an acetone series and embedded in Spurr's resin. Ultrathin sections were cut with a diamond knife and contrasted with 2% methanolic uranyl acetate and Reynolds lead citrate. Images were captured with a Hitachi T-7600 electron microscope operating at 80 kV with an AMT digital camera. Transmission electron microscopy of selected samples was performed by an experienced electron microscopist.

**Serology.** Serum samples collected from the cattle infected with strains Ondangwa and Shawawa were tested by the complement fixation test in the USDA APHIS laboratory at Days 0, 24 and 42 post-infection. Cattle infected with strain Gladysdale were not tested during this experiment, but animals endobronchially infected with this strain typically seroconvert between Days 7-14 post-infection[96] (APHIS, unpublished data).

## RESULTS

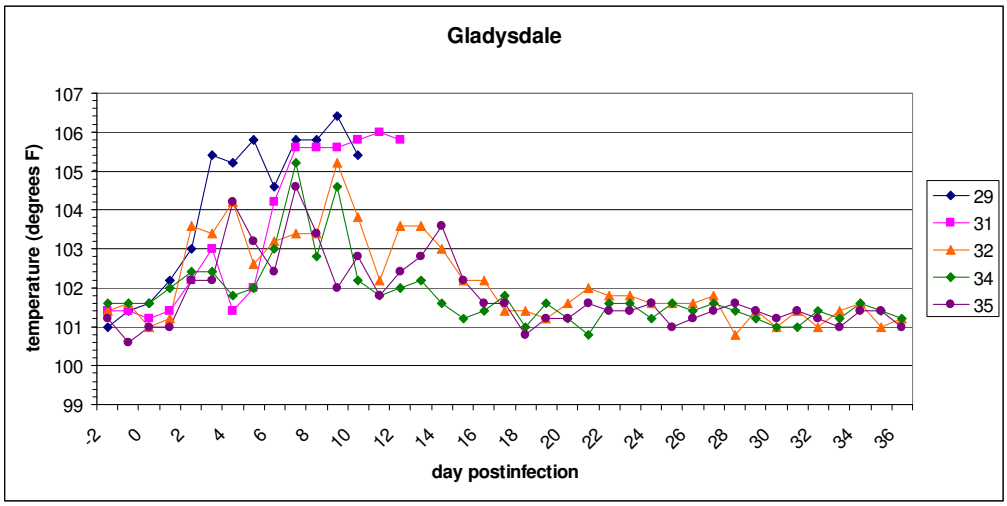
**Clinical observations.** Cattle infected with strain Gladysdale were the first to show clinical signs. Two animals exhibited increased temperatures (>103$^{o}$F) by Day 2 postinoculation, and all but one were febrile by Day 4 (**Figure 2**). One animal did not become febrile until Day 6, and this animal (#34) never exhibited a fever of above 105$^{o}$F. In animals that survived, temperatures returned to normal by Day 15 post-infection.

Two Gladysdale-infected animals developed increased respiratory rates by Day 3 post-infection. One animal became inappetent on Day 5, two days after it started showing fever of >105$^{o}$F. A second animal became inappetent on Day 6. By Day 7 both of these animals had developed cough and lethargy, and by Day 9 both were standing with abducted elbows and had increased respiratory effort. One animal had observable weight loss by Day 7, although the isolation facilities prohibited actually weighing the cattle. Unilateral right-sided pleurodynia was evident by Day 9 in both animals. One of these animals developed joint effusion in a tibiotarsal joint on Day 7, and was grade 4/5 lame on the limb (toe-touching lame). One animal demonstrated intermittent open-mouth breathing on Day 9. The two severely-affected animals (#29 and #31) became moribund and were euthanized on Days 10 and 13 post-infection respectively (**Table 2**).

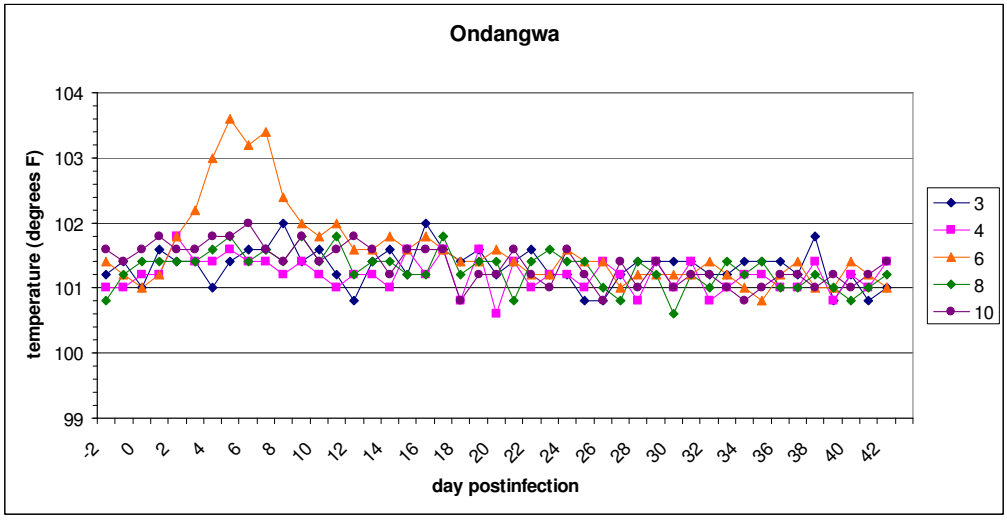
The three less-severely-affected Gladysdale-infected animals developed increased respiratory rates by Day 8, but never became inappetent. Two exhibited mild unilateral right-sided pleurodynia. No open-mouth breathing, increased respiratory effort, altered stance or lameness were observed. Two of the three showed intermittent cough between Days 9-14. One animal showed no clinical signs of illness except slightly increased respiratory rate and moderate fever, and these resolved by Day 10.

In animals infected with strain Ondangwa, only one animal exhibited a mild increase in temperature (102.6-103.6$^{o}$F) between Days 4-7. Two animals showed mild cough and slightly increased respiratory rates between Days 7-12. One animal infected with strain Shawawa showed fever between 102.6-103.6$^{o}$F on Days 4-6 only. This animal also exhibited a mild cough between Days 6-9. No other clinical signs consistent

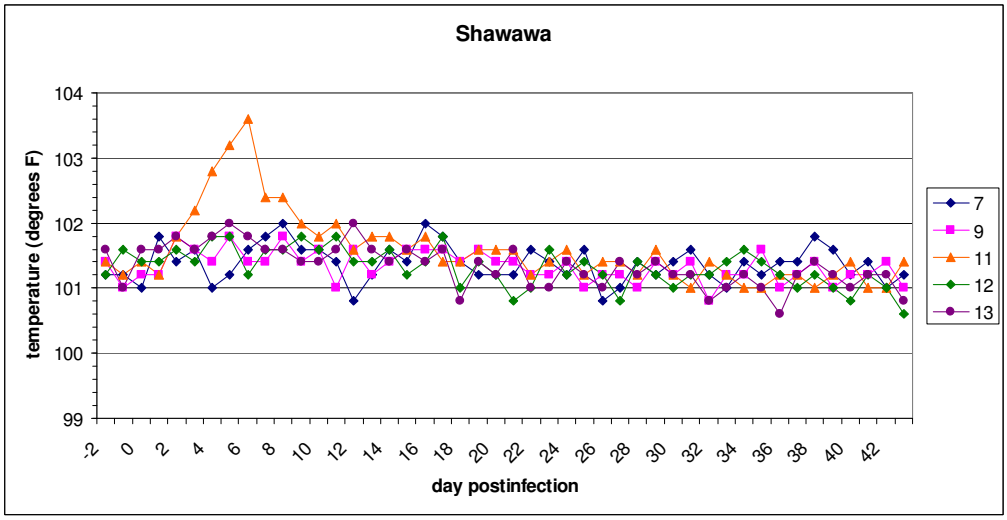
**Figure 2. Rectal temperatures of animals infected with MmmSC.** A. strain Gladysdale; B. strain Ondangwa; C. strain Shawawa. Two Gladysdale-infected animals were euthanized prior to the conclusion of the experiment due to severe disease.


Gladysdale
temperature (degrees F)
day postinfection
29
31
32
34
35

A


Ondangwa
temperature (degrees F)
day postinfection
3
4
6
8
10

B


Shawawa
temperature (degrees F)
day postinfection
7
9
11
12
13

C

**Table 2. Clinical results of infection with MmmSC.** Fifteen naïve cattle were endobronchially infected with one of three strains of MmmSC and followed through euthanasia and postmortem. Animals annotated as "died" were euthanized *in extremis*. ↑LN = enlarged tracheobronchial lymph nodes; BIP = bronchointerstitial pneumonia; PP = pleuropneumonia.

| Animal | Strain | Clinical Signs | Outcome | Postmortem |
|---|---|---|---|---|
| 7 | Shawawa | None | survived | None |
| 9 | Shawawa | None | survived | None |
| 11 | Shawawa | Fever, cough | survived | Mild chronic focal BIP |
| 12 | Shawawa | None | survived | Minimal chronic focal BIP |
| 13 | Shawawa | None | survived | Mild chronic focal BIP |
| 3 | Ondangwa | Cough, tachypnea | survived | Severe chronic focal BIP, ↑LN |
| 4 | Ondangwa | None | survived | Minimal subacute focal BIP, ↑LN |
| 6 | Ondangwa | Fever, cough, tachypnea | survived | Severe chronic focally extensive BIP, pleural adhesions, ↑LN |
| 8 | Ondangwa | None | survived | ↑LN |
| 10 | Ondangwa | None | survived | Moderate chronic focal BIP, ↑LN |
| 29 | Gladysdale | Fever, cough, tachypnea, dyspnea, lameness, inappetence, pleurodynia, open-mouth breathing | died (Day 10) | Pleural effusion and adhesions, severe extensive PP and BIP, synovitis, pericarditis, ↑LN |
| 31 | Gladysdale | Fever, cough, dyspnea, tachypnea, inappetence, pleurodynia, weight loss | died (Day 13) | Pleural effusion and adhesions, severe extensive PP and BIP, pericarditis, ↑LN |
| 32 | Gladysdale | Fever, cough, tachypnea, mild pleurodynia | survived | Focal extensive PP and BIP, sequestrum, ↑LN |
| 34 | Gladysdale | Fever, tachypnea | survived | Mild focal PP |
| 35 | Gladysdale | Fever, cough, tachypnea, mild pleurodynia | survived | Focal extensive PP and BIP, sequestrum, ↑LN |

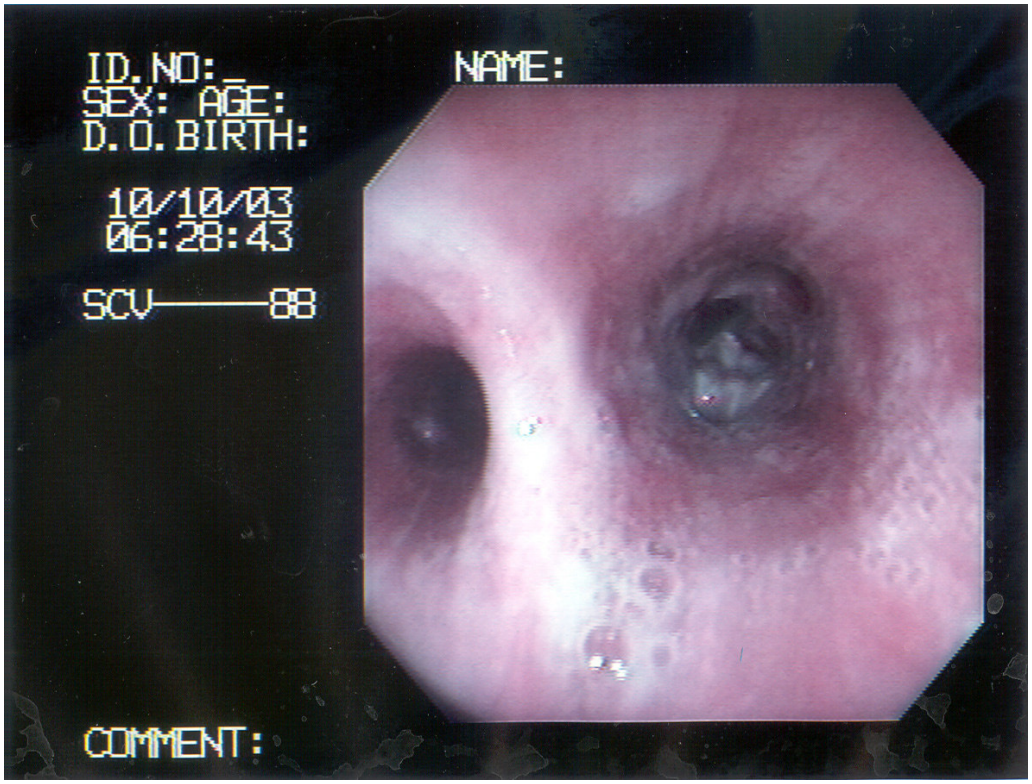



**Figure 3. Endoscopy, Ondangwa, Day 3 post-infection.** Endoscopic view of a right caudodorsal bronchus. Note the foamy mucus in the foreground and thicker secretions deeper in the bronchus.

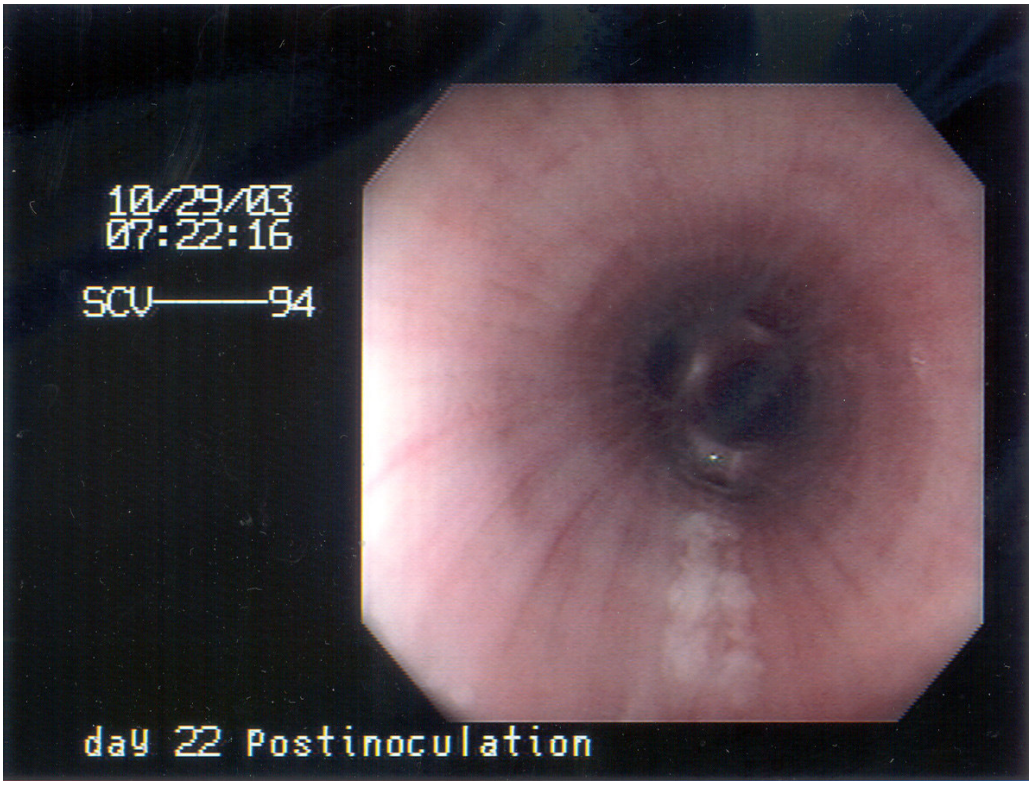



**Figure 4. Endoscopy, Ondangwa, Day 22 post-infection.** Right caudodorsal lung. Minimal exudate is visible on the ventral surface of this bronchus.

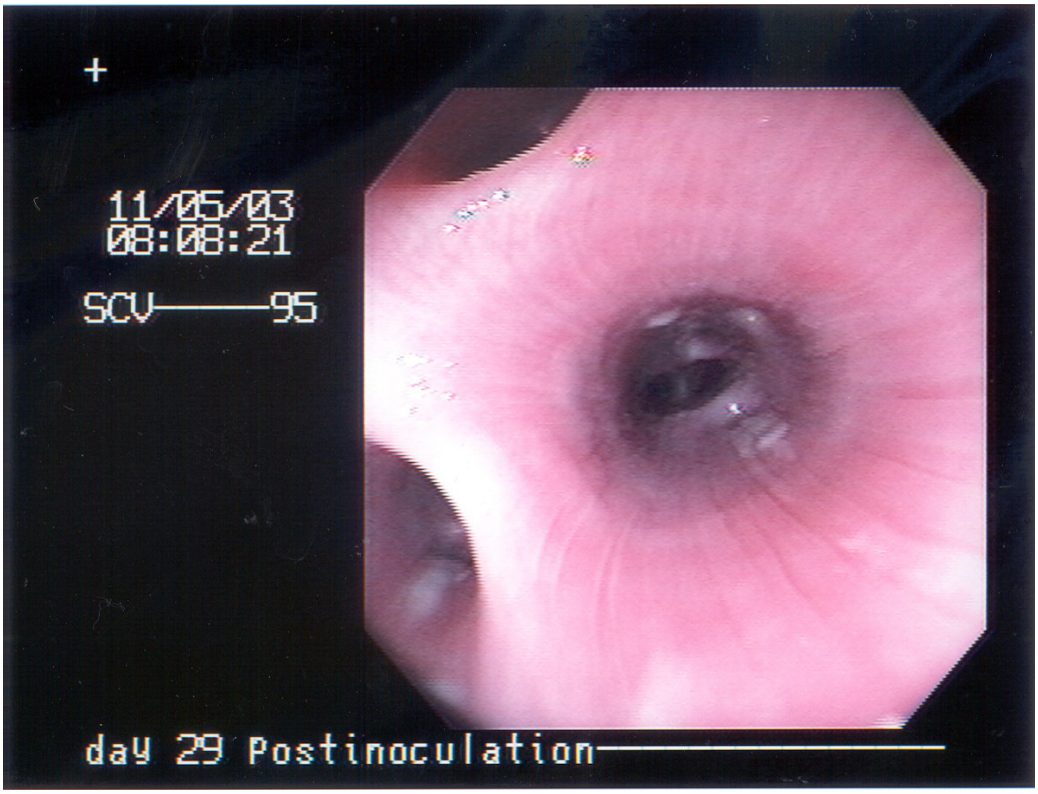



**Figure 5. Endoscopy, Ondangwa, Day 29 post-infection.** Right caudodorsal lung. A small amount of exudate remains visible in the bronchi.

with CBPP were seen in either the Ondangwa or Shawawa groups. Endoscopic views of the affected areas of lung in all three groups of cattle revealed minimal evidence of lung pathology; only mild increases in lung secretions were seen post-inoculation (**Figures 3-5**).

**Gross pathology.** Two animals infected with strain Gladysdale were euthanized when they became moribund. These animals demonstrated severe unilateral pleural effusion; severe, locally extensive unilateral, chronic, fibrinous pleural pneumonia; and moderate, multifocal, coalescing bronchopneumonia with fibrinoeffusive, proliferative pleuritis (**Figure 6**). These two animals also showed moderate, subacute, pericardial effusion; marked, interstitial peritracheal, perivascular, mediastinal and sternal edema; and marked lymphadenomegaly and edema of the tracheobronchial lymph nodes. One of these cattle also showed moderate, diffuse, subacute, fibrinous synovitis and fibrinohemorrhagic and necrotic tenosynovitis of the right rear tibiotarsal joint (**Figure 7**).

The three Gladysdale-infected cattle that were euthanized on Day 36 post-inoculation had varying degrees of pulmonary lesions. Two cattle had marked to severe focally extensive, caudal to caudodorsal, chronic pleuropneumonia with sequestrae and focal coalescing mucopurulent to caseous abcesses and severe, focally extensive, chronic, proliferative pleuritis (**Figures 8-11**). The third animal had mild, focal, unilateral, chronic broncho- and interstitial pneumonia. One animal had mild synovial proliferation of a tibiotarsal joint. In all cases, CBPP lesions were on the right side of the pleural

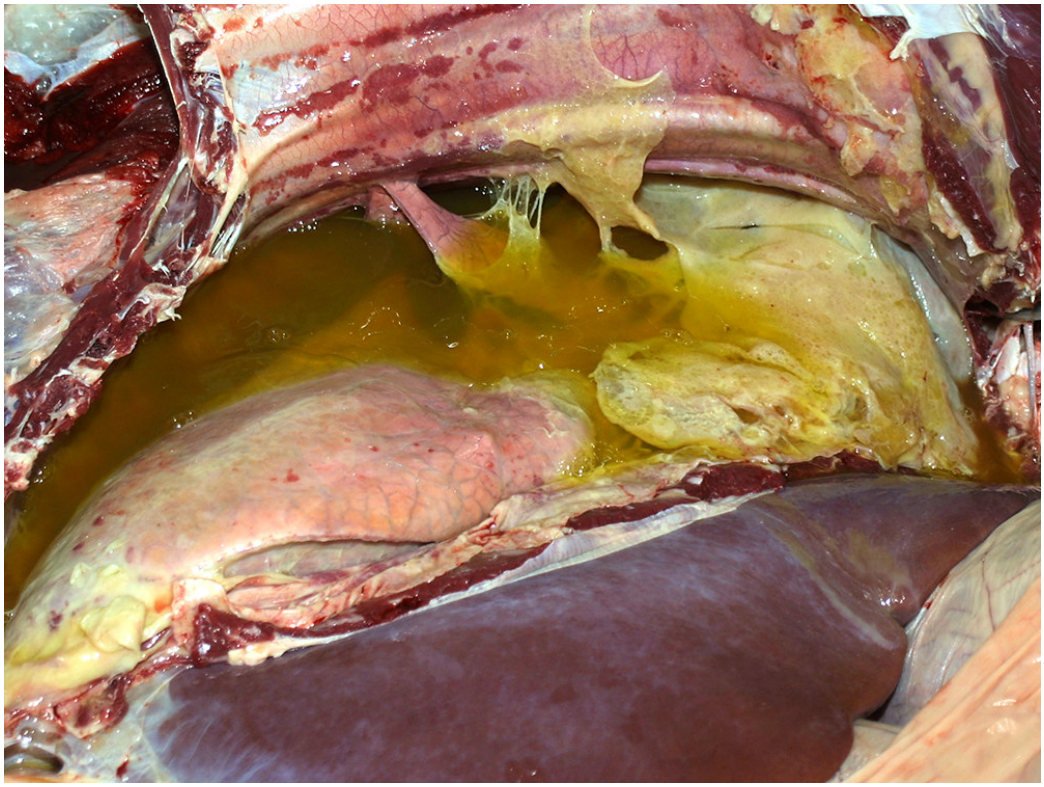


**Figure 6. Gladysdale, 10 days post-infection, right thoracic cavity.** Severe, unilateral, chronic, fibrinoeffusive pleuropneumonia. Dorsal is to the left, cranial is to the top of the photo. The thorax was filled with large quantities of a translucent yellow fluid containing strands and sheets of fibrin, the latter of which were adherent to the parietal and visceral pleura. In areas of the caudal dorsal lung the lobes were adherent to the diaphragm, rib cage and adjacent lobes of the lung. The parietal pleura was multifocally covered by a velvet-like, hyperemic proliferative mesothelium.

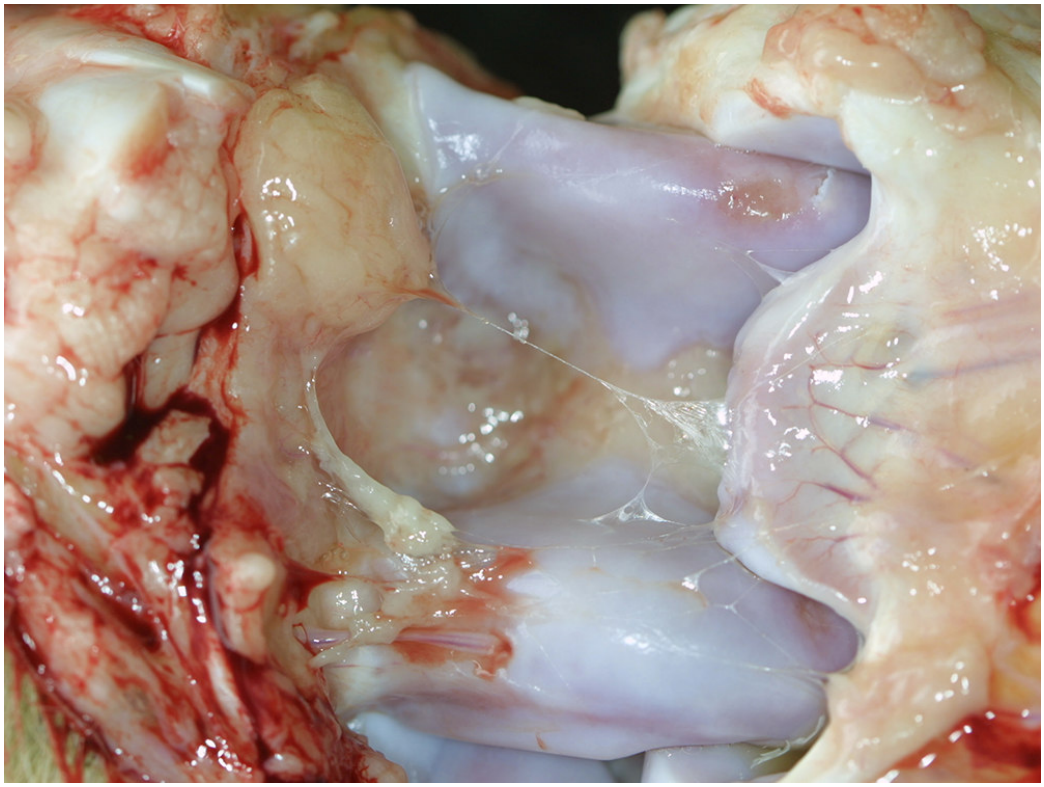


**Figure 7. Gladysdale, 13 days post-infection, right tibiotarsal joint.** Mild, diffuse, fibrinous arthritis/synovitis. The joint contained a watery synovial fluid with flecks and strands of fibrin. The synovial membranes were slightly hyperemic.

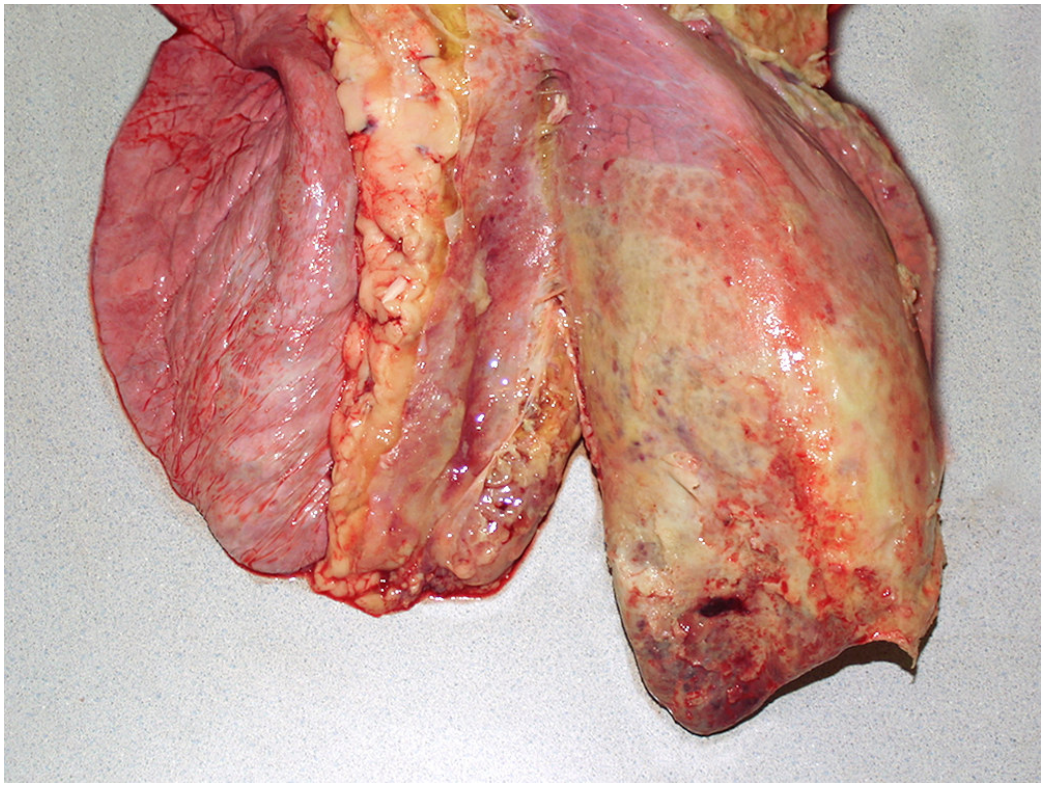


**Figure 8. Gladysdale, 36 days post-infection, bilateral lungs and mediastinum.** Severe, unilateral, chronic, fibrinous pleuropneumonia. The right caudal lung was severely enlarged, firm and covered by a sheet of fibrin. Further cranial on the right caudal lobe there is externally visible widening of interlobular septae (edema). The left lung was unaffected. Note also the the widening of the mediastinum, which was due to edema and a marked increase in the size of the mediastinal lymph nodes, which were 5-10X normal in size and edematous.

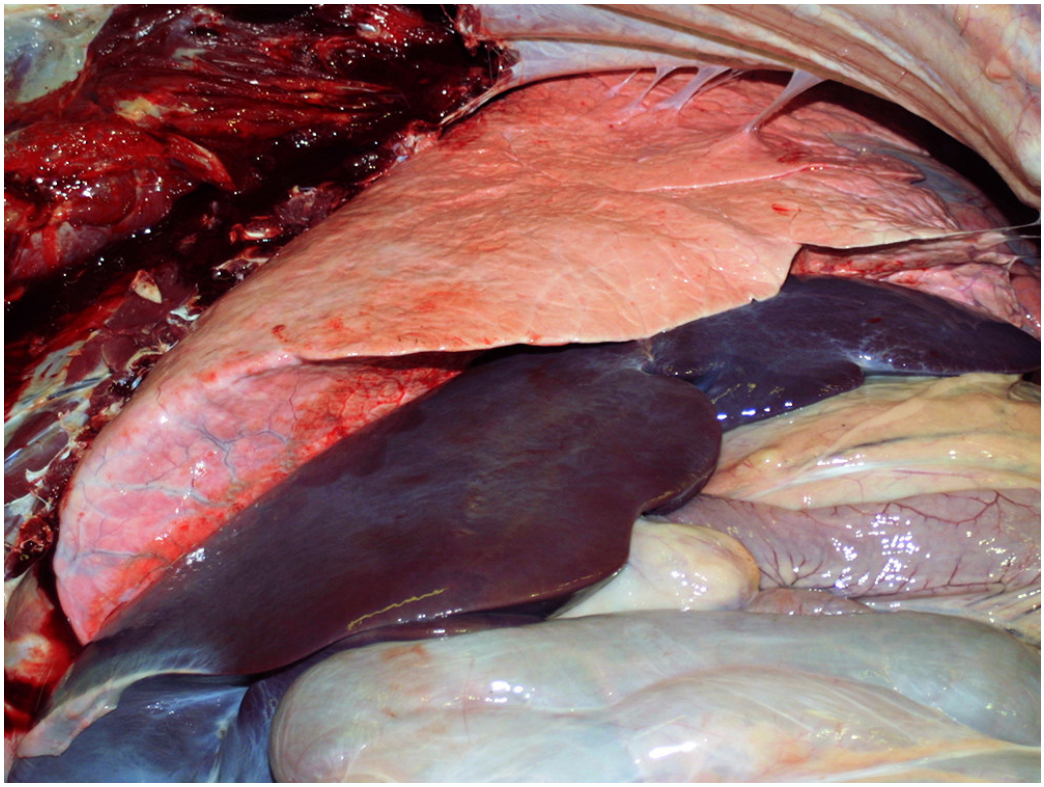


**Figure 9. Gladysdale, 36 days post-infection, right thoracic cavity.** In the right caudal lung the interlobular septae were widened, the lung margins were rounded and the lung, on palpation, was firm with a palpable discrete mass. The fibrous adhesions in the cranial lung were evidence of past non-CBPP-associated anteroventral bronchopneumonia.

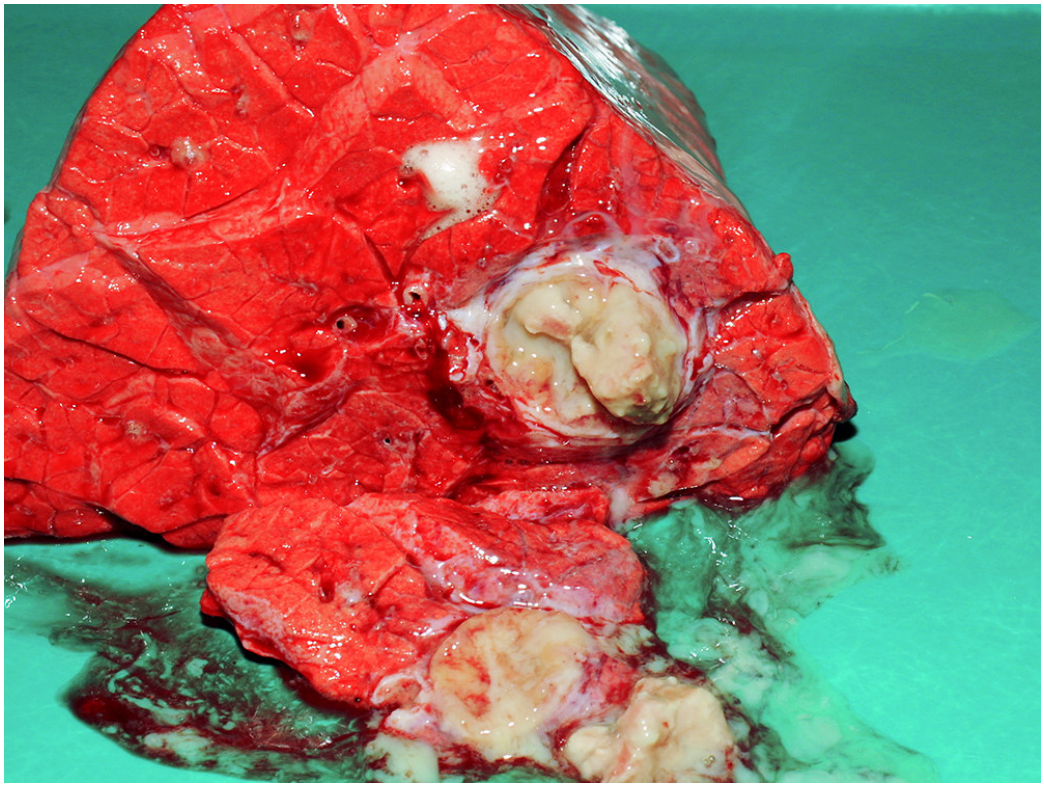


**Figure 10. Gladysdale, 36 days post-infection, right caudal lung lobe.** A mass was observed on cross section. The mass was well-demarcated and contained a mucopurulent to caseous core (abscess).

**Figure 11. Gladysdale, 36 days post-infection, cross section of right caudal lung.** Severe, unilateral, chronic, pleuropneumonia with sequestrum formation. In the right caudal lung was a well-delineated focus of sequestered lung in which the lung parenchyma was dark red, firm and contained foci of necrosis. In the surrounding aerated lung there is marked widening of interlobular septae (edema) and proliferation of fibrous connective tissue (marbling).

**Table 3. Scoring algorithm for gross and histologic lesions in infected cattle.** Gross Pathology: Percentile of lung involvement. Histopathology: Percentile of parenchymal involvement in sections of representative lesions. Scoring of gross and histopathology is based on degree of severity. This is generally a matter of % of parenchymal involvement and is a subjective score. The pathology (distribution, duration, infiltrate/exudate) is consistent in most cases.

| **Gross Pathology Scores** | **Score** | **Percentile** | | **Histopathology Scores** | **Score** | **Percentile** |
|---|---|---|---|---|---|---|
| **No Gross Lesions** | **0** | **0** | | **No Significant Lesions** | **0** | **0** |
| **Minimal** | **1** | **<5** | | **Minimal** | **1** | **<5** |
| **Mild** | **2** | **5 - 10** | | **Mild** | **2** | **5 - 10** |
| **Moderate** | **3** | **10-25** | | **Moderate** | **3** | **10-25** |
| **Marked** | **4** | **25-50** | | **Marked** | **4** | **25-50** |
| **Severe** | **5** | **>50** | | **Severe** | **5** | **>50** |

cavity and in the right lung, consistent with the site of inoculation. Two animals also exhibited evidence of resolved anteroventral bronchopneumonia. The average gross pathology score for CBPP lung lesions in the Gladysdale group was 4 of 5 (**Table 3**), and 1.8 of 5 for lesions of the lymph nodes. The lymph nodes of the two nonsurviving cattle had an average score of 4.5 of 5 and the three surviving cattle (which were euthanized and necropsied more than 20 days after resolution of clinical signs) had lymph node scores of 0 of 5.

Four of five cattle infected with strain Ondangwa had characteristic gross lesions of CBPP at necropsy on Day 42 post-infection. All lesions attributable to CBPP were located in the right caudal lung at the site of inoculation. Lesions included minimal to severe, focally extensive, unilateral, subacute to chronic, broncho- and interstitial pneumonia. One animal had chronic pleural adhesions attributable to CBPP. Again, several cattle had evidence of resolved anteroventral bronchopneumonia separate from any CBPP lesions, and lesions of chronic verminous pneumonia (lungworm) were also seen. No animals had gross lesions of the lymph nodes, and no joint involvement was noted. The average gross lung lesion score for animals infected with strain Ondangwa was 2.8 of 5, and the average lymph node score was 0 of 5.

Three cattle infected with the Shawawa strain had characteristic gross CBPP lesions at necropsy on Day 43 post-infection, also confined to the right caudal lung. These lesions included minimal to mild, focal, unilateral, subacute to chronic broncho- and interstitial pneumonia. No Shawawa-infected cattle had lesions of the lymph nodes or joints. As in the other groups, several animals had evidence of resolved anteroventral

bronchopneumonia or verminous pneumonia unrelated to CBPP. The average gross lung lesion score for cattle infected with strain Shawawa was 0.5 of 5 and the average lymph node score was 0 of 5.

**Histopathology.** Microscopic lesions of the lung in Gladysdale-infected cattle included marked to severe, diffuse, chronic, suppurative bronchopneumonia with bronchial lymphoid hyperplasia in all animals (**Figures 12-21**). Lesions of the tracheobronchial lymph nodes ranged from mild lymphoid hyperplasia to marked, diffuse lymphoid hyperplasia with sinus histiocytosis and edema (**Figures 22-23**). Joint lesions in three cattle demonstrated mild to marked diffuse, subacute, proliferative lymphocytic synovitis. Two animals had no microscopic joint involvement. The average histopathology lung lesion score in Gladysdale-infected cattle was 4.6 of 5 and the average lymph node lesion score was 3.6 of 5.

Cattle infected with MmmSC strain Ondangwa demonstrated moderate to severe, focally-extensive to diffuse, chronic, suppurative interstitial and bronchopneumonia with bronchial lymphoid hyperplasia (**Figure 24**). Lesions of the lymph nodes included moderate to marked diffuse, lymphoid hyperplasia of the tracheobronchial lymph nodes. No joint lesions were noted. The average microscopic lung lesion score was 3.2 of 5 and the average lymph node score was 3.4 of 5.

Shawawa-infected cattle had lung lesions ranging from mild, diffuse, chronic, bronchial associated lymphoid hyperplasia to marked, diffuse, chronic, interstitial pneumonia with bronchial associated lymphoid hyperplasia to severe, diffuse, chronic

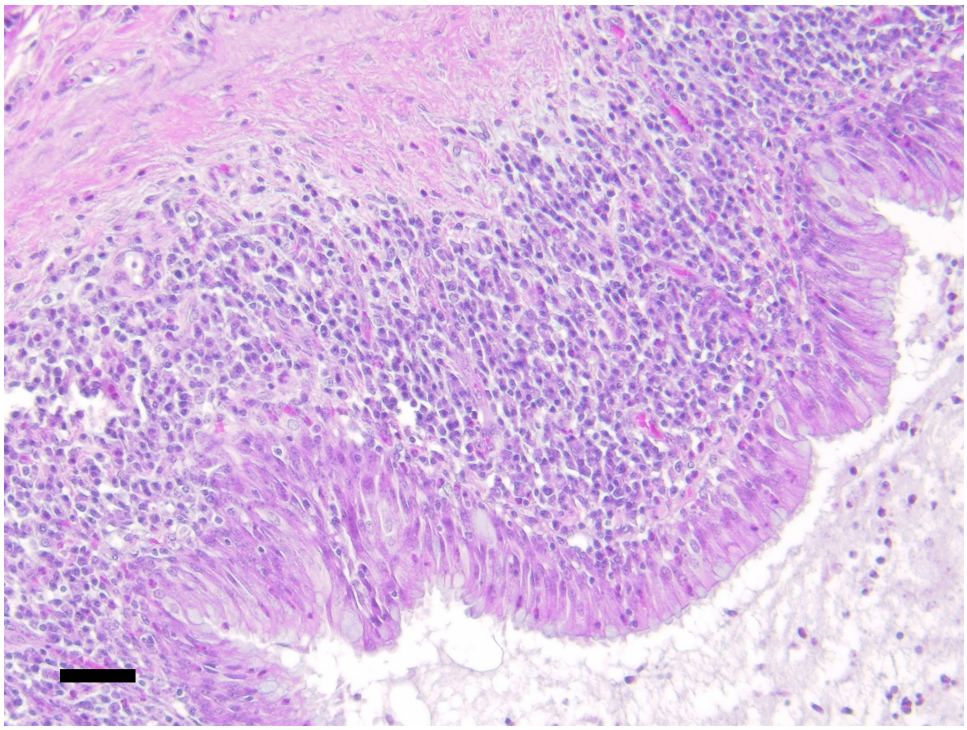


**Figure 12. Gladysdale, 13 days post-infection, right lung.** Severe, unilateral, chronic, lymphoproliferative bronchopneumonia. The lamina propria-submucosa of the airways (bronchioles) were infiltrated by large numbers of lymphocytes and plasma cells and lesser numbers of macrophages and fewer neutrophils. Magnification: 400x; bar = 150µm.

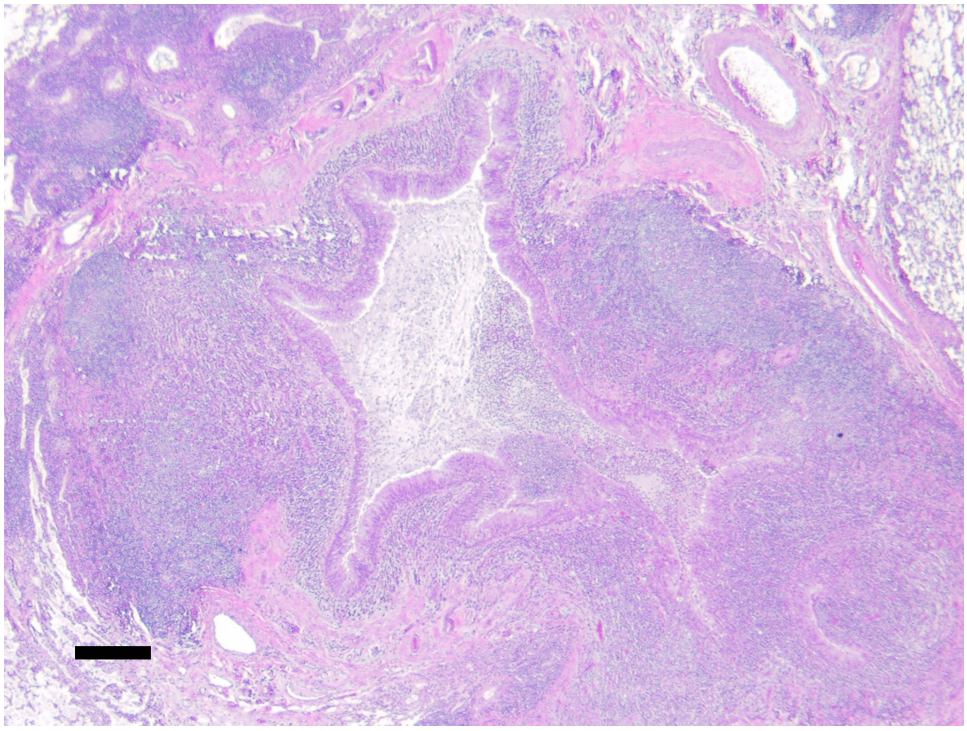


**Figure 13. Gladysdale, 13 days post-infection, right caudal lung bronchiolitis.**

Severe, unilateral, chronic, lymphoproliferative bronchopneumonia and bronchiolitis.

Magnification: 100x; bar = 1500 mm.

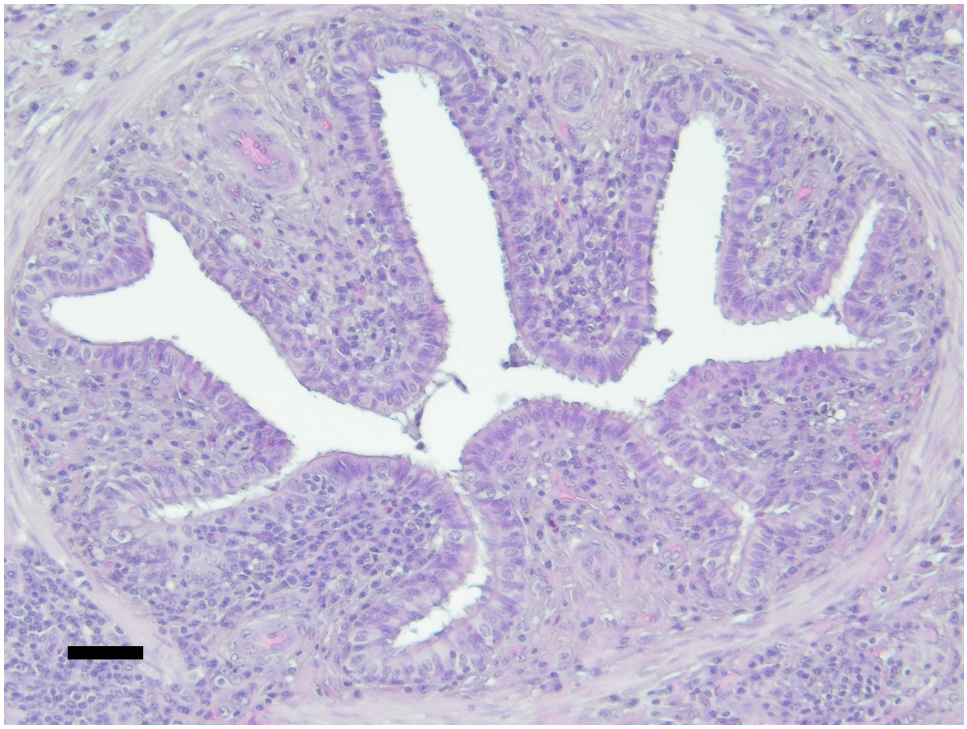


**Figure 14. Gladysdale, 13 days post-infection, bronchus of right lung.** Severe, unilateral, chronic, lymphoproliferative bronchopneumonia. The lamina propria-submucosa of the airways (bronchioles) were infiltrated by large numbers of lymphocytes and plasma cells and lesser numbers of macrophages and fewer neutrophils. Magnification: 400x; bar = 150µm.

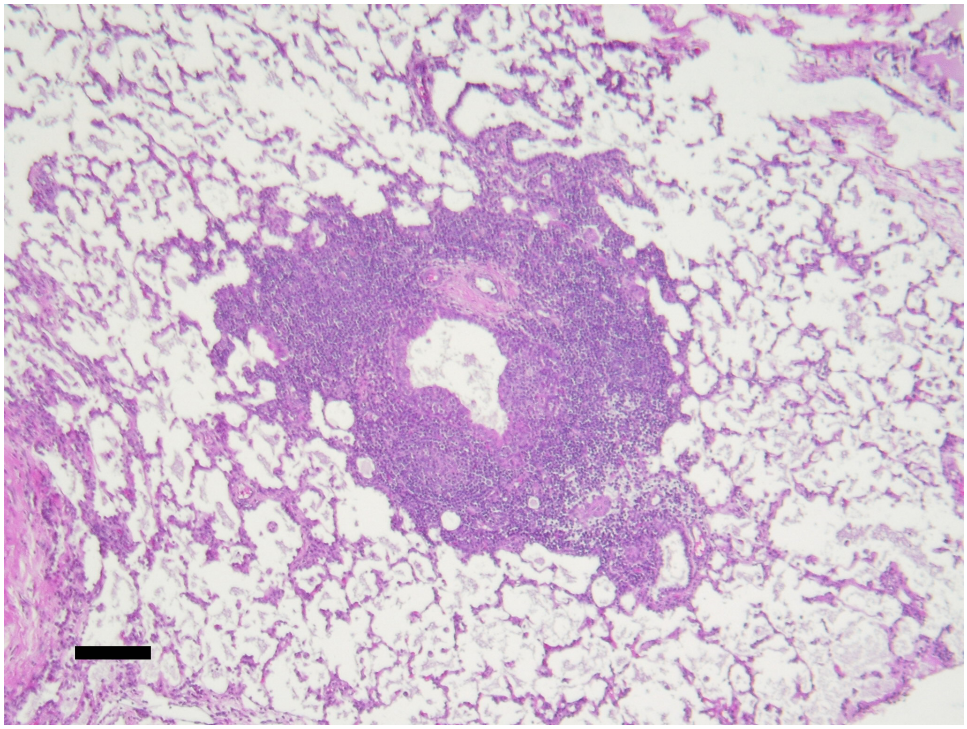


**Figure 15. Gladysdale, 13 days post-infection, right lung bronchus.** Severe, unilateral, chronic, lymphoproliferative bronchopneumonia/ bronchiolitis. The lamina propria-submucosa of the airways (bronchioles) were infiltrated by large numbers of lymphocytes and plasma cells and lesser numbers of macrophages and fewer neutrophils. Magnification: 100x; bar = 1500 µm.

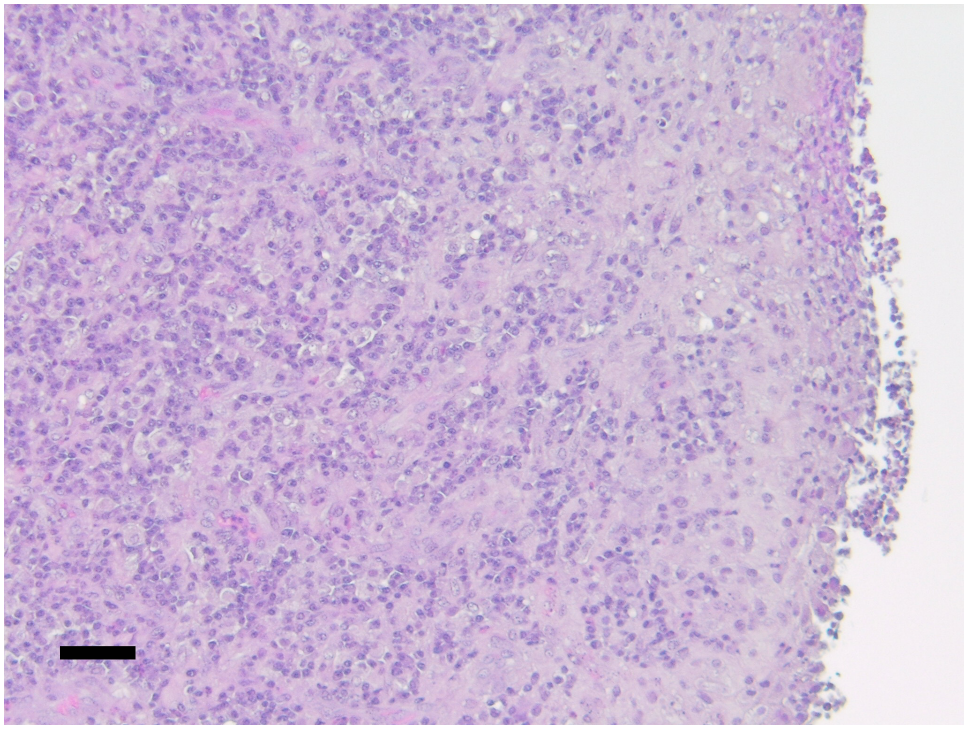


**Figure 16. Gladysdale, 36 days post-infection, right lung.** Severe, unilateral, chronic, lymphoproliferative pleuropneumonia. The visceral pleura, which was effaced and replaced by fibrin and fibrous connective tissue, was markedly thickened and infiltrated by large numbers of lymphocytes, plasma cells and lesser numbers of foamy macrophages and rare neutrophils. Magnification: 400x; bar = 150 µm.

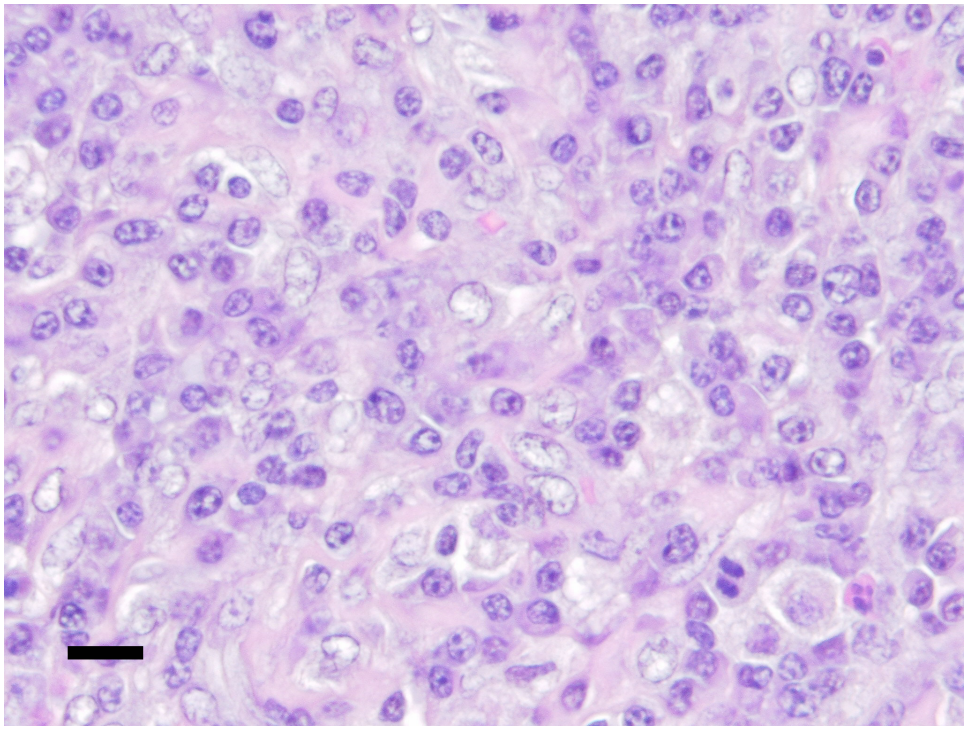


**Figure 17. Gladysdale, 36 days post-infection, right lung, lymphoproliferation.**

Severe, unilateral, chronic, proliferative pleuropneumonia. Magnification: 1000x; bar = 25 µm.

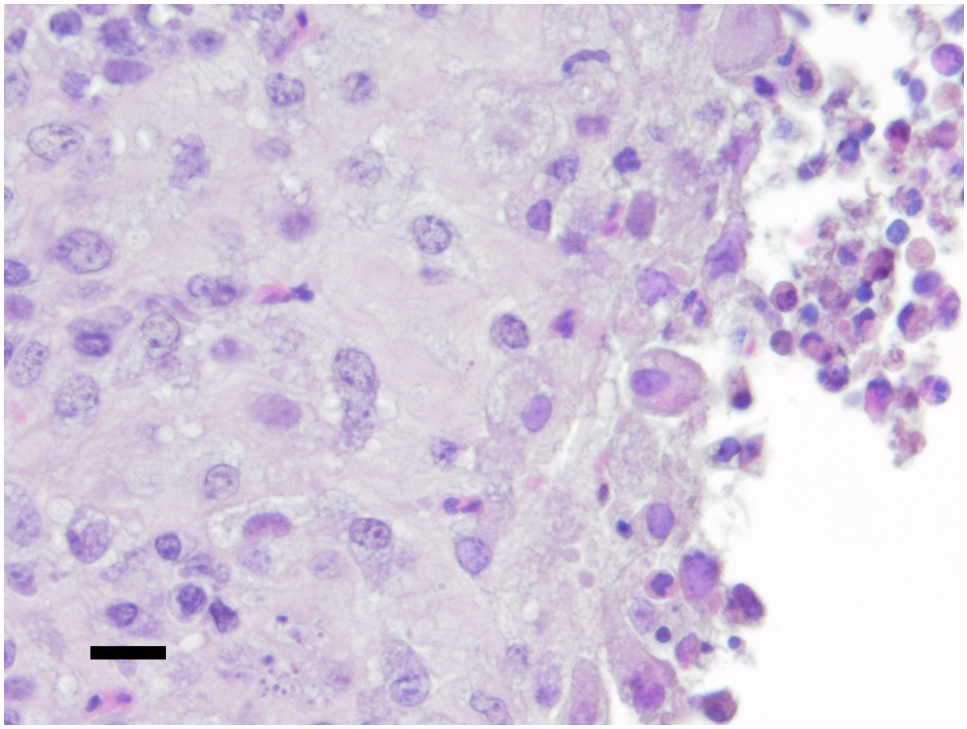


**Figure 18. Gladysdale, 36 days post-infection, pleura of right lung.** Severe, unilateral, chronic, lymphoproliferative pleuropneumonia. The visceral pleura was effaced and replaced by fibrous connective tissue, and was markedly thickened and infiltrated by large numbers of lymphocytes, plasma cells, foamy macrophages and neutrophils. Magnification: 1000x; bar = 25 µm.

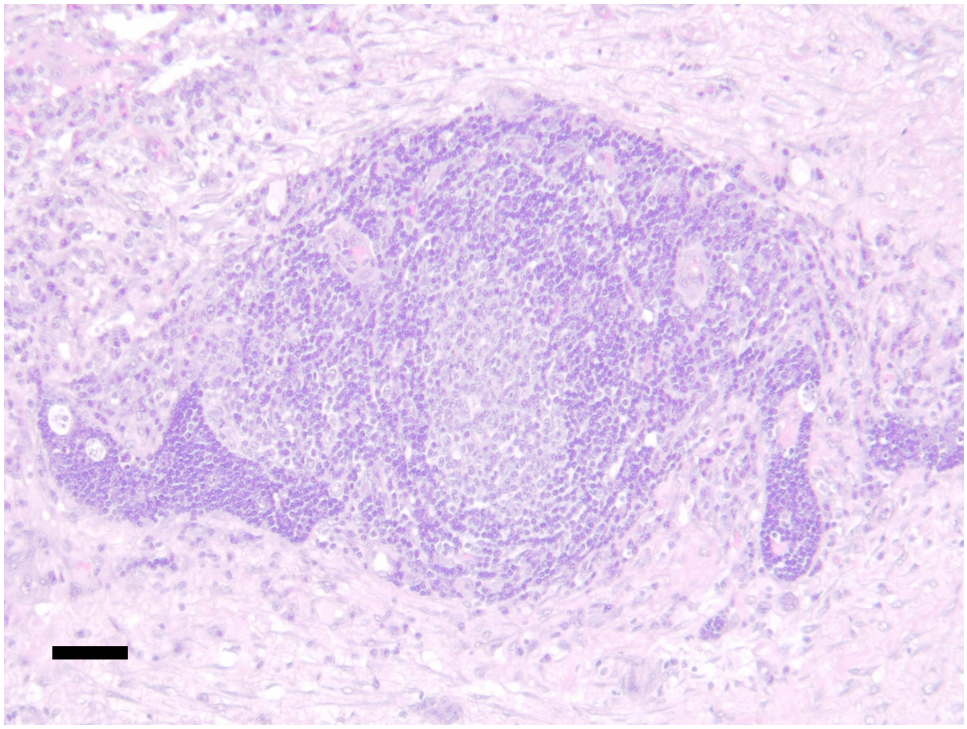


**Figure 19. Gladysdale, 36 days post-infection, right lung lymphoid follicle.** The lung was replaced with massive interlobular fibrosis with lymphoid proliferation and lymphoid follicular development. Magnification: 400x; bar = 150 µm.

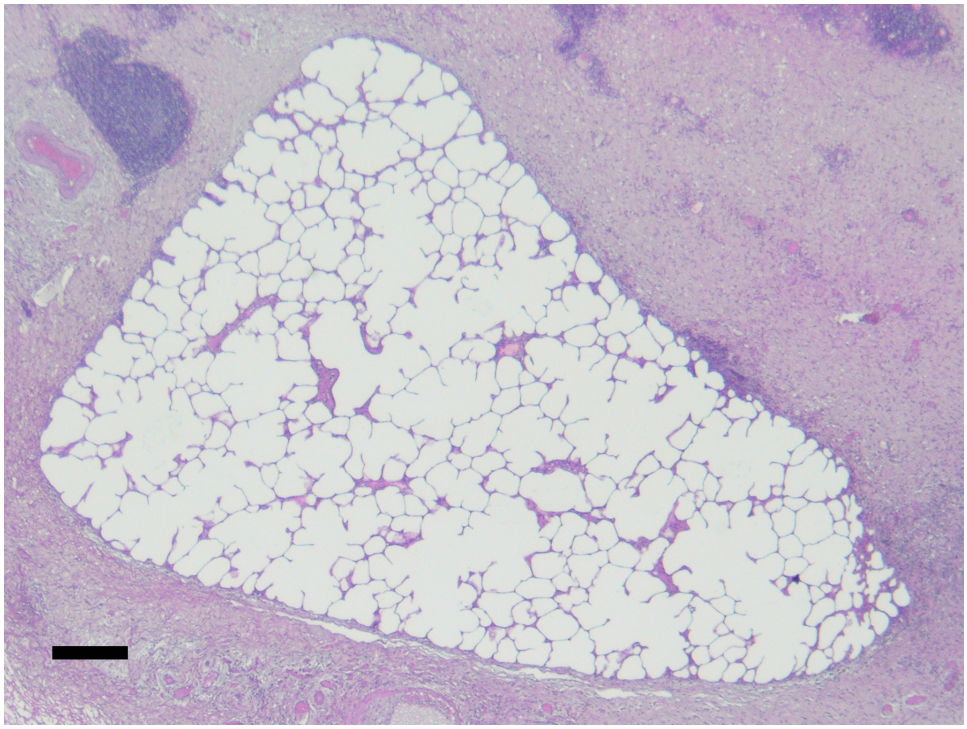


**Figure 20. Gladysdale, 36 days post-infection, right lung fibrosis.** Severe, unilateral, chronic, fibrinoproliferative pneumonia. The lung was often characterized by "marbling" with pronounced interlobular fibrosis with sequestration of pulmonary tissues accompanied by lymphoid proliferation with follicular development. Magnification: 40x; bar = 1000 µm.

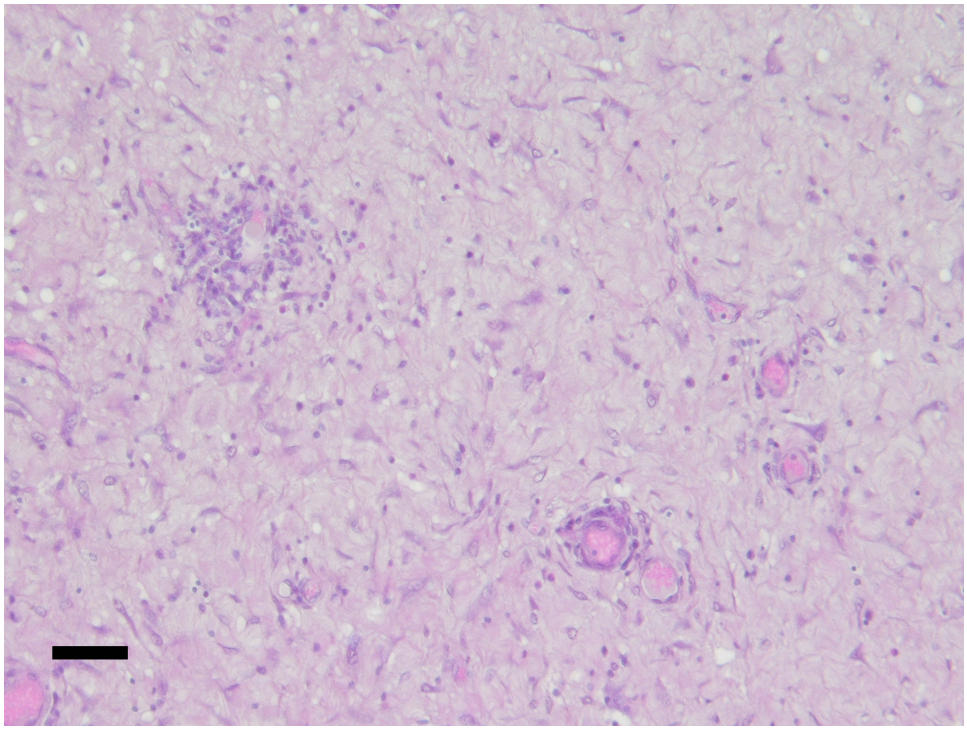


**Figure 21. Gladysdale, 36 days post-infection, right lung, fibrous connective tissue.**

Massive dense interlobular fibrosis occurred, sequestering pulmonary tissue.

Magnification: 400x; bar = 150 µm.

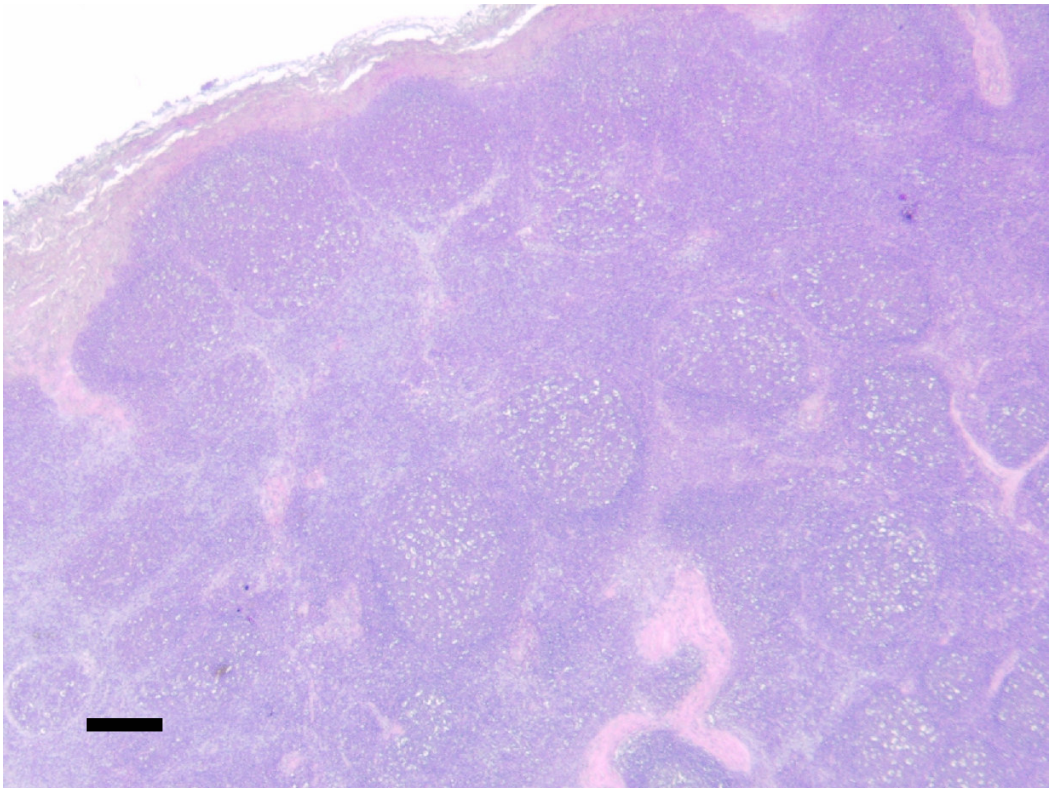


**Figure 22. Gladysdale, 13 days post-infection, tracheobronchial lymph node.** Lymphoid germinal centers with expanding mantles were markedly increased in number, indicative of an expansion of the B cell compartment. Magnification: 100x; bar = 1500 µm.

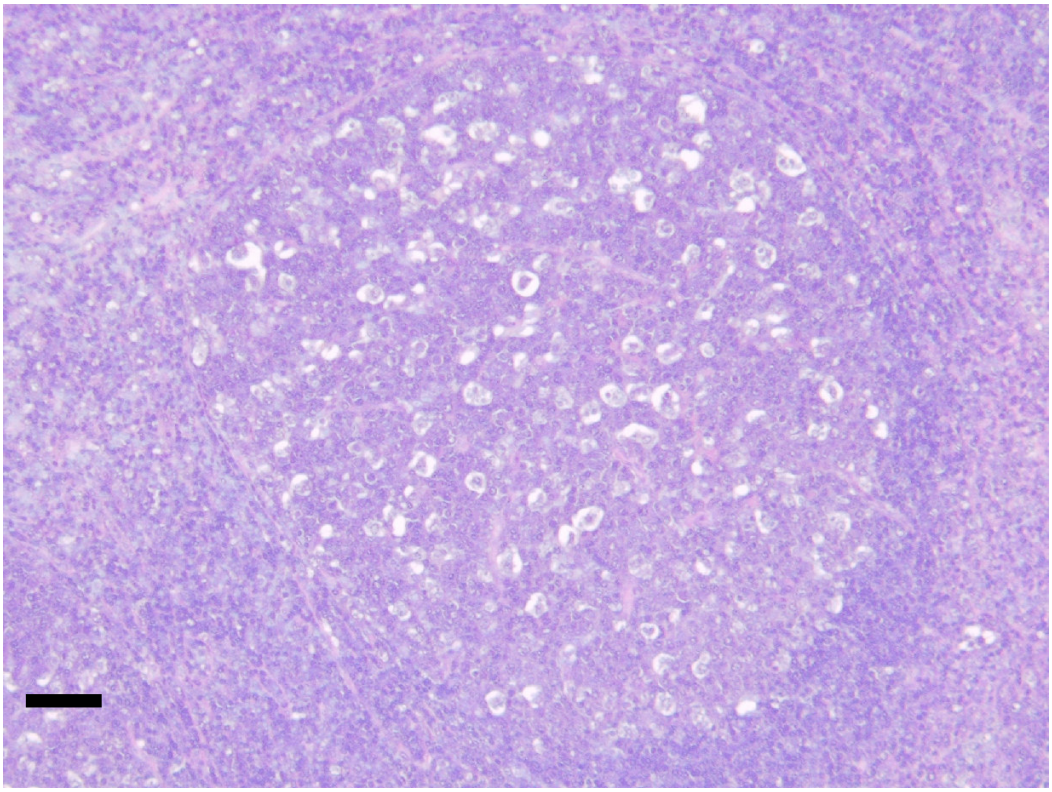


**Figure 23. Gladysdale, 13 days post-infection, lymph node germinal center.**

This tracheobronchial lymph node showed a germinal center containing extensive evidence of apoptosis of lymphoblastoid cells. Magnification: 400x; bar = 150 μm.

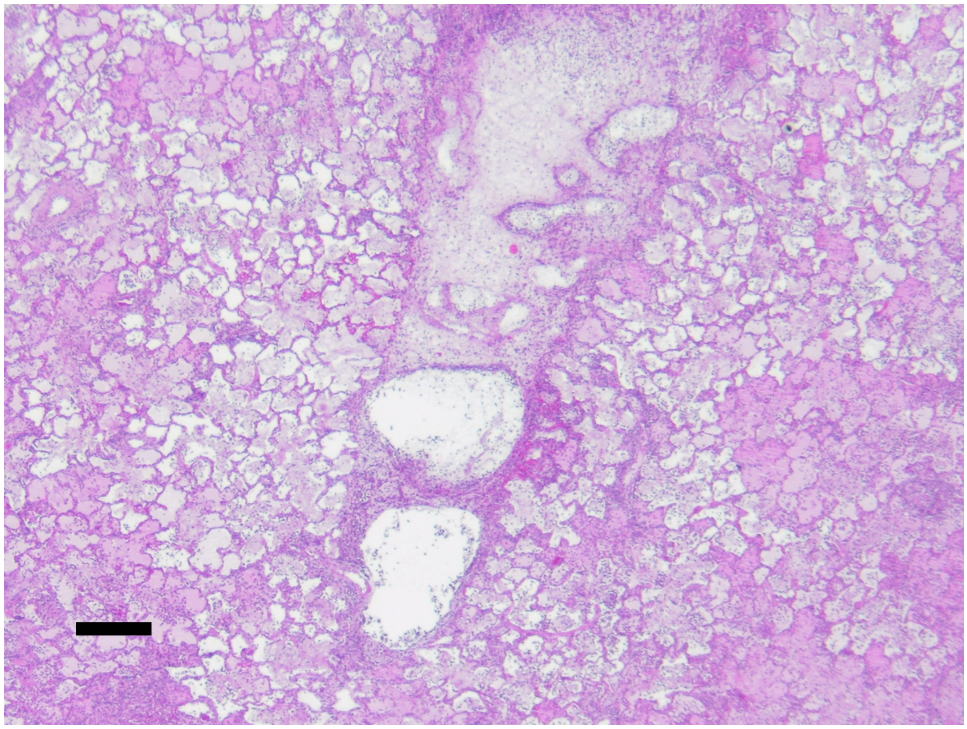


**Figure 24. Ondangwa, 42 days post-infection, pneumonia of right lung.** Subacute sero-fibrinous pneumonia, necrosuppurative and edematous. Magnification: 40x; bar = 1000 µm.

bronchiolitis obliterans with severe bronchial associated lymphoid hyperplasia (**Figure 25**). Lesions of the lymph nodes in this group were moderate to marked with lymphoid hyperplasia of the tracheobronchial lymph nodes. No joint lesions were noted. The average microscopic lung lesion score in this group was 2.6 of 5 and the average lymph node score was also 2.6 of 5.

**Transmission electron microscopy.** TEM was successful only on Gladysdale animals at necropsy. Samples taken via bronchial biopsy contained excessive crush artifact. Images of the bronchial tissues collected at necropsy showed the mycoplasma present in large numbers on the epithelial surface, but none were seen within the epithelial cells (**Figure 26**). Mycoplasmas were present in high concentrations at the base of the cilia of respiratory epithelial cells.

**Serology.** All animals infected with strains Ondangwa and Shawawa were negative via the complement fixation test at Days 0, 24 and 42 post-infection.

## DISCUSSION

One isolate (Gladysdale) used in this study was selected as it was known to produce severe acute pulmonary disease in *Bos taurus* cattle. The Ondangwa and Shawawa isolates were African field strains known to produce moderate-to-severe disease in *Bos indicus* cattle. We anticipated a varied spectrum of disease in each experimental group. The clinical results supported the selection of these strains to produce the desired models, as Gladysdale-infected cattle showed significantly more

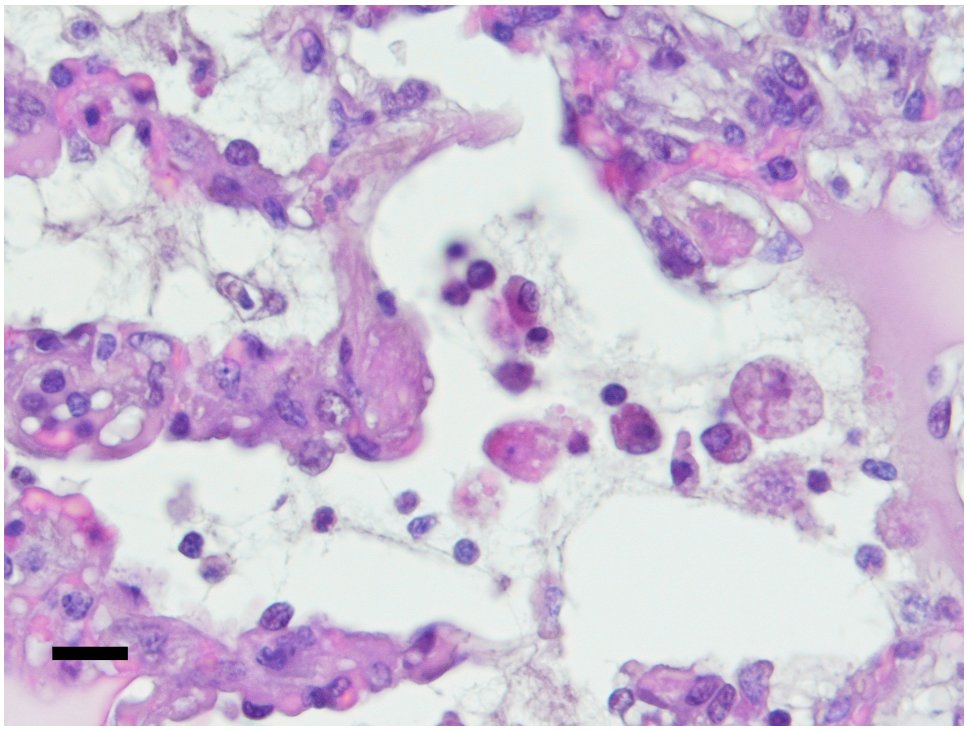


**Figure 25. Shawawa, 43 days post-infection, right lung.** Chronic fibrinoeffusive pneumonia with thickened alveolar septae and intra-alveolar lymphocytes and macrophages. Magnification: 1000x; bar = 25 µm.

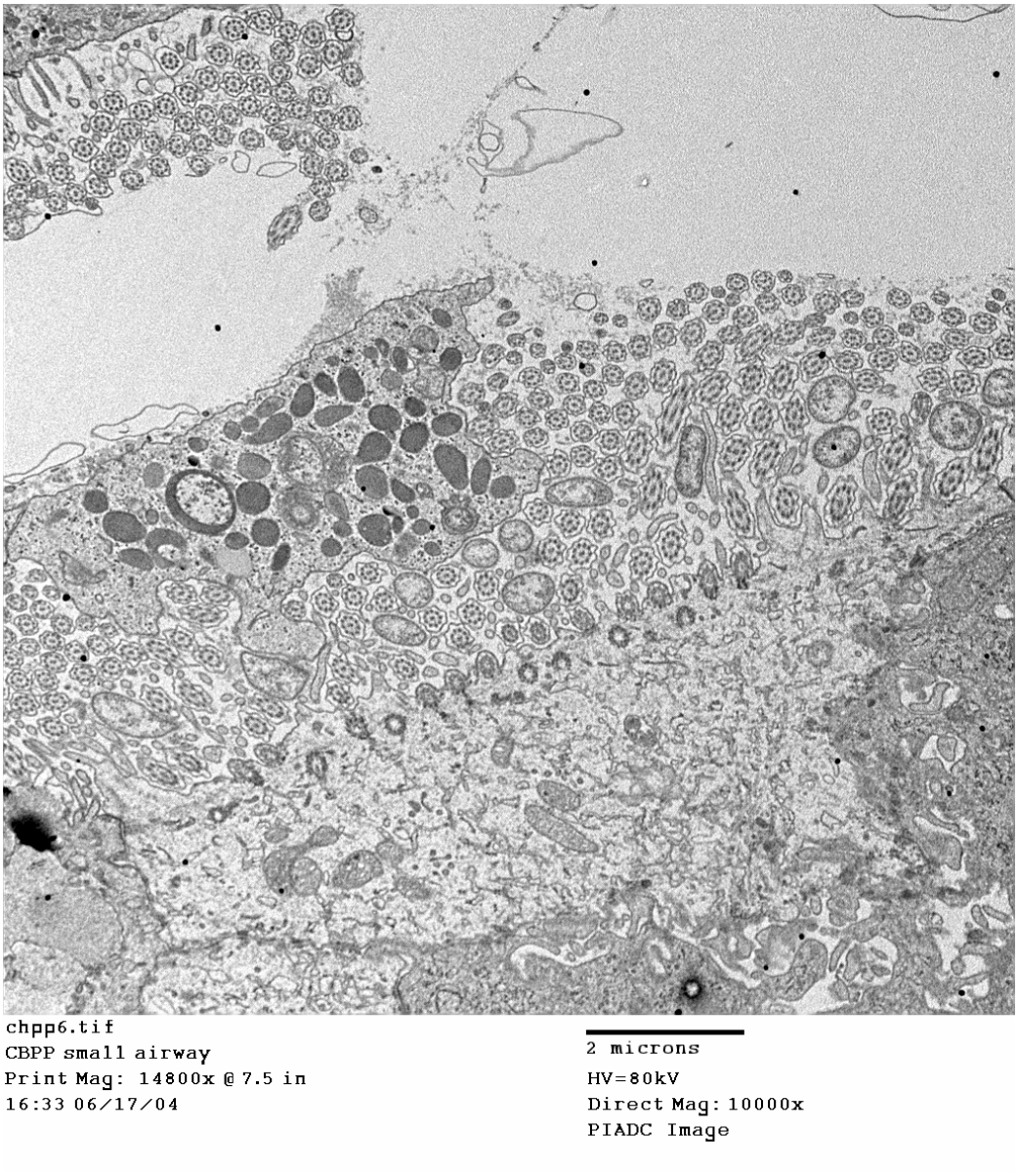



**Figure 26. Gladysdale, 36 days post-infection, transmission electron microscopy.**

A natural killer cell adjacent to mycoplasma among the cilia of a bronchial epithelial cell. Magnification: 14,800X; bar = 2 µm.

severe clinical signs of disease than did Ondangwa or Shawawa-infected animals. While the number of animals used was small, the results support previous reports as far as the percentage of animals demonstrating clinical recovery that still had lesions at necropsy[17].

The onset of clinical disease was rapid in all models compared to natural infection, with the first clinical signs appearing as soon as Day 3 post-inoculation. The incubation period of naturally-occurring disease varies between 5-207 days, but is normally between 31-45 days[17]. Animals infected with strain Gladysdale showed significantly more severe clinical disease than did animals infected with either of the two African field strains, and onset of characteristic clinical signs was faster by 3-4 days in Gladysdale-infected animals. Gladysdale-infected cattle also demonstrated higher rectal temperatures, with all animals but one reaching >105$^{o}$C within nine days of inoculation. In comparison, one each of Ondangwa and Shawawa-infected animals reached a maximum of 103.6$^{o}$C, only a mild febrile response. The other Ondangwa- and Shawawa-infected cattle remained afebrile, although as temperatures were taken only once daily a rapidly-spiking and resolving fever could not be ruled out. Fever is an unreliable indicator of infection, however, as demonstrated by the three Ondangwa and two Shawawa animals without detectable fever that nonetheless had gross necropsy lesions of CBPP.

All Gladysdale-infected cattle but one developed cough between Days 7-10 of infection, and the cough generally lasted less than a week. Three Ondangwa animals showed coughing lasting 5-7 days. Only a single Shawawa animal developed a cough, which was evident for only 4 days. Cough would appear to be an inconsistent clinical

sign, as animals without noticeable cough had lesions of moderate to severe disease at necropsy. Increased respiratory rate was present in most animals starting within the first week post-infection, but no appreciable difference was noted between groups.

The spectrum of gross necropsy lesions differed between groups of cattle, with the two Gladysdale-infected cattle requiring early euthanasia demonstrating profound pleural effusion and fibrin formation in addition to extensive lung involvement. The three Gladysdale-infected animals that survived until the end of the experiment had minimal pleural effusion despite two having demonstrated mild pleurodynia earlier in the course of infection. As neither ultrasound examination of the thorax or thoracocentesis was done, it is unknown whether these animals had pleural effusion earlier in the course of infection that had resolved by the time of necropsy. Two of these three Gladysdale-infected cattle did develop sequestrae encapsulated with a thick fibrous layer after only 36 days of infection. In contrast, Ondangwa- and Shawawa-infected cattle failed to develop classical sequestrae after 42 days of infection despite demonstrating other gross lesions consistent with CBPP. It is unknown whether Ondangwa- or Shawawa-infected animals would develop sequestrae given a longer time course, as neither of these strains had been evaluated previously under controlled conditions and no published reports of the natural outbreaks involving these isolates exist. The apparent failure of Ondangwa- and Shawawa-infected cattle to form sequestrae is apparently related to their virulence. No reports exist comparing long-term shedding of MmmSC in cattle infected with sequestra-forming or putatively non-sequestra-forming strains. In fact, very little is known regarding the kinetics of long-term shedding of any isolate of MmmSC as most

experiments, like this study, were either short-term or did not evaluate mycoplasmal shedding in the long term[53,65,69,97].

Histopathology of lesions from infected animals had the full spectrum of CBPP lesions. Microscopic lung lesions from Ondangwa- and Shawawa-infected animals were often quite similar to those from Gladysdale-infected cattle, but the percentage of affected lung was less. All cattle with CBPP lesions, regardless of strain, demonstrated chronic interstitial and suppurative bronchopneumonia with fibrosis. Lung tissue, particularly the airway submucosa, had profound infiltration of lymphocytes and plasma cells with fewer macrophages and occasional neutrophils. Differences between strains were mostly evident as percentage of parenchymal involvement in the lesions evaluated. Virulence between strains appeared to be related to the amount of lung parenchyma each isolate was able to influence rather than the degree of lesions within the affected parenchyma. Notably, Gladysdale-infected cattle had a greater degree of fibrosis and bronchial lymphoid hyperplasia than did either Ondangwa- or Shawawa-infected cattle, with development of lymphoid follicles within lung parenchyma. The increased fibrosis in Gladysdale-infected animals may reflect that strain's tendency to create sequestrae, while strains Ondangwa and Shawawa were unable to elicit the same response in the host and did not form sequestrae.

Lesions of the tracheobronchial lymph nodes varied between strains, with Gladysdale-infected cattle demonstrating marked lymphoid hyperplasia, edema, and sinus histiocytosis. Lymph node germinal centers in Gladysdale-infected animals that succumbed to CBPP had numerous apoptotic lymphoblastoid cells that were not seen in

Gladysdale-infected animals that survived infection or in Ondangwa- or Shawawa-infected animals. Cattle infected with Ondangwa and Shawawa had less prominent lymphoid hyperplasia of tracheobronchial lymph nodes, and edema was absent.

Only Gladysdale-infected cattle had microscopic joint lesions of lymphocytic synovitis, and two of the three cattle with joint lesions were the animals euthanized early in the experiment. Two animals with microscopic synovitis had never been noted to be lame or to have clinical joint effusion. One animal had histologic evidence of synovitis at Day 36 necropsy, long after all clinical signs of disease had resolved. No joint lesions were seen in either Ondangwa- or Shawawa-infected cattle; it is undetermined if lesions might have been present earlier in infection but resolved by Day 42 necropsy.

Transmission electron microscopy of bronchial biopsies taken during weekly sampling was unsuccessful due to excessive crush artifact of the tissue. Images taken from necropsy tissue demonstrated large numbers of mycoplasma colonizing the surface of respiratory epithelial tissue, often predominantly at the base of the cilia. These images do not show any evidence of intracellular MmmSC, but the intimacy of contact between the mycoplasma and the epithelial cell could not be definitively determined. Most mycoplasma remain extracellular when infecting a host animal, but certain species are capable of invasion and intracellular survival in non-phagocytic cells. The most-studied member of this class is *M. penetrans*, but *M. fermentans*, *M. pneumoniae*, *M. genitalium* and *M. gallisepticum* have all been shown to survive intracellularly under some conditions[98]. *Mycoplasma penetrans* has been shown to have fibronectin-binding ability[99] and to alter the host cell cytoskeleton[100], both characteristics of invasive bacteria.

Other mycoplasma, most notably *M. fermentans*, are attributed to have the ability to fuse with the host cell. This property is apparently dependent on the unesterified cholesterol content of the mycoplasma cell membrane[101]. No information has been published on whether MmmSC possesses fusogenic capability, although *M. capricolum*, also within the mycoides cluster, is known to be fusogenic[101].

The negative results of the complement fixation test on the Ondangwa and Shawawa animals at Days 24 and 42, conducted by the official United States testing laboratory of the USDA Animal and Plant Health Inspection Service, serve to underline the weaknesses of that test. The CFT is reported to detect only approximately 70% of chronic cases of CBPP and often fails to detect asymptomatic animals early in the course of infection[4]. Despite direct inoculation of the organism into the lung and evidence of successful infection in the form of fever in several animals, the CFT remained uniformly negative in all Ondangwa and Shawawa animals including those shown to have CBPP at necropsy. The CFT is recommended as a herd level test[102], so the small numbers of animals in our experimental groups may have decreased test sensitivity. Nonetheless, the CFT is currently the official standard for determining infection per the OIE[88,103], and further evidence of its drawbacks only underlines the need for more sensitive and specific diagnostic options.

Currently, feedlot veterinarians and animal producers in the United States are becoming concerned about the increasing incidence and severity of *Mycoplasma bovis* pneumonia in feedlot cattle (reported at the 2007 annual Forum of the American College of Veterinary Internal Medicine). In addition, these veterinarians have warned about the

potential for reintroduction of CBPP into the United States and have expressed concern that belated recognition of this disease would occur, in part because of the similarity between lesions of CBPP and severe *M. bovis* pneumonia[104-108]. Cattle shipping and feedlot management practices in the United States would provide a fertile ground for rapid transmission of CBPP, whether accidental or deliberate, and delays in diagnosis would prove extremely costly. This underscores the need for further study of CBPP and development of additional means of both diagnosis and differentiation from *M. bovis*. Better understanding of the mechanisms by which MmmSC causes disease may indirectly provide clues for the management of *M. bovis* pneumonia as well.

In conclusion, direct endobronchial inoculation of $10^{10}$ MmmSC without adjuvants appears to be a reliable and repeatable model of CBPP infection. We validated this model using three African/Australian cluster strains of MmmSC reported to have differing virulence. Two of these strains, Ondangwa and Shawawa, have not previously been evaluated in experimental models. Results from this model support descriptions of the relative virulence of natural infections involving these isolates: Gladysdale exhibited the greatest virulence, followed by Ondangwa, and Shawawa demonstrated the lowest virulence. Using this infection model, this study generated an acute severe disease group (Gladysdale) and two chronic inapparent disease groups of differing severity (Ondangwa and Shawawa). Morbidity and incidence of necropsy lesions for each inoculated strain were comparable to reports of naturally-occurring disease. The model produced lower mortality rates than those reported in natural infections of each isolate, although the number of cattle infected with each isolate was small. Lack of mortality in the

Ondangwa- and Shawawa-infected groups as compared to natural outbreaks may be due to differences in susceptibility between *Bos taurus* and *Bos indicus* cattle.

Advantages of endobronchial inoculation include a known site of infection within the lung which may be repeatedly sampled as needed, and relatively atraumatic lower respiratory tract sampling in standing cattle. Disease onset was more rapid than for in-contact infection, compressing the required number of sampling days and allowing all cattle to be sampled as a cohort with a defined time since infection. This model is also adaptable to field conditions: although a videoendoscope was used in this study, a portable light source and visual (rather than video) confirmation of endoscope placement would be equally effective. However, either sampling method requires at least 3 personnel and a means of cattle restraint such as a chute and headgate. Other disadvantages of this model include expensive endoscope equipment requirements, fragility of equipment, and labor.

Standardization of inoculum size and placement, time since infection and infection site in experimental cattle should remove much of the uncertainty present in the studies of natural or experimental in-contact CBPP infections. MmmSC strain differences may be more readily determined without the confounding factors of unknown bacterial dose and indeterminate time since infection. The development of these three side-by-side CBPP models of differing virulence will also permit more direct comparison of host effects versus inoculum effects, as the model, using any of the tested strains, could be applied to discrete subgroups of cattle segregated by species, breed or BoLA

type. This model and the data presented here should facilitate future study of MmmSC strains exhibiting differential virulence.

# CHAPTER III

# TEMPORAL GLOBAL GENE EXPRESSION PROFILES OF CATTLE INFECTED WITH *MYCOPLASMA MYCOIDES MYCOIDES* SMALL COLONY

## INTRODUCTION

Little is known about the interactions of MmmSC with the bovine host. Previous studies have focused on particular aspects of the immune system[53,65-67,97], and have primarily measured soluble cytokines. A significant proportion of the published studies on CBPP have used *ex vivo* or *in vitro* models, which likely do not duplicate the complexity of the interactions in the host animal. It is known that MmmSC causes mycoplasmemia, indicating its global effects in the host. The clinical signs in infected cattle, along with the previously published studies, support the intense involvement of the immune system in this disease[53,65,69,97]. The normal manner of infection is direct contact, with the respiratory system being the route of infection[4]. This study will investigate the temporal gene expression profiles in several respiratory-associated tissue types of cattle infected with CBPP. The working hypothesis is that there will be differential gene expression of multiple genes and associated cellular pathways between unexposed and challenge infected cattle at several time points post-infection. Analysis of the global host transcriptome will identify differentially-expressed genes as targets for later experiments and potentially provide insight into the manner in which MmmSC interacts with the host animal. This study holds the possibility of the identification of possible targets for

continued investigation of the host immune response. This research is unique in that no published studies exist investigating the *in vivo* host gene expression of cattle with CBPP.

## MATERIALS AND METHODS

**Animals, isolates, route of infection and sampling procedures.** Animals, route of infection and endoscopic sampling procedures were described in detail in Chapter II. This experiment was conducted only with MmmSC strain Gladysdale (APHIS).

**Isolation of total RNA from bronchoalveolar lavage fluid.** BAL fluid was aliquoted into microcentrifuge tubes immediately after collection and centrifuged at room temperature and 12,000 x *g* for 10 minutes. Supernatant was discarded and pellets resuspended in 2ml RNAlater (Ambion). Tubes were stored at 4ºC until further processing. For RNA extraction, pellets were centrifuged at 12,000 x *g* for 10 minutes at 4ºC and the supernatant was discarded. Pellets were resuspended in 1ml Tri-reagent (Molecular Research), and RNA extracted per manufacturer's instructions. The resultant RNA pellet was re-suspended in nuclease-free water (Ambion). Contaminant genomic DNA was removed by RNase-free DNase I treatment (Ambion) according to the manufacturer's instructions, and samples were stored at -80ºC until used. RNA concentration was determined via NanoDrop® ND-1000 (NanoDrop) spectrophotometry.

**Isolation of RNA from bronchial biopsies.** Two 2.5mm biopsy samples were obtained via endoscopic biopsy forceps from a bronchial bifurcation deep in the right caudal lung near the site of inoculation. The samples were placed into RNAlater and stored at 4ºC until processing. During processing, biopsies were minced into small pieces

with a sterile scalpel, and placed in Tri-Reagent (2 biopsy samples / 1 ml of reagent) and further homogenized with a tissue grinder. RNA was extracted according to the Tri-Reagent manufacturer's instructions. The resultant RNA pellet was re-suspended in nuclease-free water (Ambion). Contaminant genomic DNA was removed by RNase-free DNase I treatment (Ambion) according to the manufacturer's instructions, and samples were stored at -80ºC until used. RNA concentration was determined via NanoDrop® ND-1000 (NanoDrop) spectrophotometry.

**Isolation of RNA from nasal cytology brushes.** Cytology brushes were amputated into a microcentrifuge tube containing 2ml RNAlater immediately after collection and stored at 4C until further processing. Brushes were scraped into the RNAlater using a sterile scalpel blade, then the tube was centrifuged at 12,000 x *g* for 10 minutes at 4ºC. The supernatant was discarded and the pellet resuspended in 1ml Tri-Reagent. Processing continued as described above.

**RNA isolation from whole blood.** Ten milliliters of whole blood was collected into a syringe, then the needle removed and the blood added to a conical tube containing 30ml Tri-Reagent BD (Molecular Research) and mixed thoroughly. Blood was stored at 4ºC until further processing. RNA was extracted according to the manufacturer's protocol, and processing continued as described above.

**Preparation of bovine reference RNA.** Total RNA was isolated from Madin-Darby bovine kidney (MDBK) and bovine B lymphocyte (BL-3) cell lines (ATCC) and fresh bovine brain using the Tri-Reagent protocol. Cell lines were grown in 150 $cm^2$ cell culture flasks with minimum essential medium Eagle (MEME) (ATCC) supplemented

with 10% heat-inactivated fetal bovine serum. Bovine brain was harvested from the cortex and cerebellum of a Holstein male calf immediately after euthanasia. The tissue was homogenized in ice-cold Tri-Reagent. The RNA from each sample was quantitated and bioanalyzed before and after pooling the samples. Total RNA isolated from three samples was pooled together in equal amounts, aliquoted and stored at -80ºC until needed.

**Construction of cDNA microarrays and annotation.** Selective unique 70-mer oligonucleotide sets representing 13,257 cattle ORFs were obtained from normalized and subtracted cattle placenta and spleen cDNA libraries and based upon the earlier cDNA array platform GPL2864 (70) and subtracted cDNA libraries created from embryonic (Day 36 and Day 64) and extra-embryonic (Day 14 to 25) tissues (NCBI libraries 15993, 15993 and 17188). Positive controls included beta actin (ACTB), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), and hypoxanthine phosphoribosyltransferase (HPRT). Exogenous spiking controls were the soybean genes chlorophyll ab binding protein (CAB), Rubisco small chain 1 (RBS1), and major latex protein (MSG). Negative controls were Cot1 DNA, genomic DNA, spotting buffer, poly-A, and water. All 70-mer oligos were printed in 150 mM phosphate buffer at 20 uM concentration in duplicate on aminosilane-coated glass slides at the W. M. Keck Center (Dr. Harris Lewin, University of Illinois at Urbana-Champaign). The oligos were annotated based on the GenBank accession number, when available.

**Sample preparation and array hybridization.** Prior to hybridization, the microarrays were denatured by steam exposure and UV cross-linked. Microarrays were

pre-treated by washing in 0.2% SDS, followed by 3 washes in distilled water and immersion in prehybridization buffer [5X SSC, 0.1% SDS; 1% BSA in 100ml of water] at 42ºC for at least 45 minutes. Immediately before hybridization, the slides were washed 4 times in distilled water, dipped in 100% isopropanol and dried by centrifugation. The labeling and hybridization procedures for host samples were an adaptation of the protocol developed by The Institute for Genomic Research [109]. Briefly, 10 µg of total RNA were reverse transcribed overnight to aminoallyl cDNA using 6 µg of random hexamer primers (Invitrogen), 0.6 µl 50X dNTPs (Invitrogen) / aa-dUTP (Ambion) mix (2:3 aa- dUTP: dTTP) and 400U Superscript III (Invitrogen). The reaction was stopped by incubating the samples with 1M NaOH at 65ºC for 15 minutes and neutralized by adding 1M HCl. Unincorporated aa-dUTPs and free amines were removed by column passage (Qiagen) and dried using a speedvac. Dried samples were re-hydrated in 0.1M $Na_2CO_3$ buffer (pH 9.0) and labeled with Cy3-ester (reference RNA) or Cy5-ester (experimental RNA) (Amersham Pharmacia Biosciences). After one hour of incubation in the dark at room temperature, uncoupled dye was removed using columns (Qiagen) and dye incorporation calculated via NanoDrop spectrophotometry. The dried, labeled cDNA samples from experimental RNA and bovine reference RNA were resuspended in 20 µl of nuclease-free water, mixed, and heated at 95ºC for 10 min followed by 10 min at 60ºC and another 10 min at 25ºC. Samples were kept at 42ºC until hybridization. Following incubation at 42ºC and immediately before hybridization, 40 µl of 2X formamide-based hybridization buffer were added to each sample. The samples were then hybridized to the custom bovine oligo array. Slides were hybridized at 42ºC for ~42 hours in a dark, humid chamber (Corning),

then washed for 10 min at 42ºC with low stringency buffer [1X SSC, 0.2% SDS] followed by two 5-minute washes in a higher stringency buffer [0.1X SSC, 0.2% SDS and 0.1X SSC] at room temperature with agitation. Slides were dried by centrifugation at 800 X *g* for 2 min and immediately scanned.

**Data acquisition and microarray data analysis.** Immediately after washing, the slides were scanned using a commercial laser scanner (GenePix 4000B). The genes represented as spots on the arrays were adjusted for background and normalized to internal controls using image analysis software (GenePixPro 6.0). Genes with fluorescent signal values below background were disregarded in all analyses. Initially, arrays were normalized against bovine reference RNA. The resulting data was analyzed using Seralogix's suite of gene expression analysis and modeling tools (Dr. Kenneth Drake, www.seralogix.com). Genes were determined to be significantly differentially- expressed based on Seralogix's Bayesian z-score method. Using this method genes are ranked and ordered according to their expression magnitudes and gene variance is computed using a Bayesian predicted variance value. The Bayesian variance is determined by using a sliding window algorithm that averages 50 variances directly on the ascending and descending ordered sides of each gene of interest. This method is used to smooth the variances across the dynamic range of intensity values. Significantly changed genes were determined with the Bayesian z-test ($p < 0.0125$). New computational tools developed by Seralogix were used for the identification of Biosignature Dynamic Bayesian Network modeling, mechanistic gene discovery and pattern/pathway recognition. Seralogix's Biosignature Analysis Framework is comprised of an integrated suite of software tools

(XManager, XConsole & XBuilder) and relational database storage specialized for management and analysis of biosignature data.

**Validation of microarray results.** Sixteen randomly-selected bovine genes with differential expression on microarray were analyzed by quantitative RT-PCR (qRT-PCR). Two micrograms of RNA from the same samples used for microarray hybridization were reverse transcribed into cDNA using TaqMan (Applied Biosystems). For relative quantitation of target cDNA, samples were run in individual tubes in a SmartCycler I (Cepheid). One SmartMix bead (Cepheid) was used for 2 x 25 µl PCR reactions along with 20 ng of cDNA, 0.2X SYBR Green I dye (Invitrogen) and 0.3 µM forward and reverse primers (Sigma Genosys) designed by Primer Express Software v2.0 (Applied Biosystems) or Primer3Plus (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (**Table 3**). For each gene tested, the individual calculated threshold cycles (Ct) were averaged among each condition and normalized to the Ct of the bovine β2-microglobulin gene from the same cDNA samples before calculating the fold change using the $\Delta\Delta C_t$ method (Applied Biosystems Prism SDS 7700 User Bulletin #2). For each primer pair, a negative control (water) and an RNA sample without reverse transcriptase (to determine genomic DNA contamination) were included as controls during cDNA quantitation. Because our analysis considered genes differentially expressed based on z-score and not on fold change, array data were considered valid if the fold change of each gene tested by qRT-PCR was expressed in the same direction as determined by microarray analysis.

## RESULTS

RNA isolated from all four bovine sample types (bronchoalveolar lavage fluid, bronchial biopsies, nasal cytology and blood) was indirectly labeled and co-hybridized against bovine reference RNA on a custom 13.2K bovine oligoarray at each sampling Day (Days preinoculation, 1, 8, 15, 22 and 29). Bioanalysis determined that bovine reference RNA was of good quality (RIN = 9.7, 28S/18S ratio = 2.1, $OD_{260/280} = 2.01$, $OD_{260/230} = 1.85$ for reference RNA). Bioanalysis was not available for experimental samples, but $OD_{260/230} > 1.82$ via spectrophotometry. When hybridized on the arrays, the bovine reference RNA generated a readable signal intensity on more than 85% of the spots on the microarray (SNR > 3SD above background) and co-hybridization with experimental samples allowed comparison of bovine gene expression profiles across all tissue types. Signal analysis of arrays revealed that Day 29 arrays contained an unacceptable signal-to-noise ratio possibly due to contamination; data from Day 29 arrays was considered invalid and eliminated from further analysis. Arrays from all other sampling days contained usable data.

**Dynamic Bayesian modeling analysis of microarray results.** We conducted an *in silico* predictive mathematical model using the host gene expression data to identify genes, gene processes and pathways important in the pathogenesis of CBPP. Specific genes and pathways are presented below for each tissue type assayed, including genes identified as mechanistic. A mechanistic gene is a gene which has the most influence/impact within its dynamic Bayesian network model in comparison to the control or other condition. The implication of a mechanistic gene is that it may be a

candidate for being a major player in the disease, and it is being temporally modulated significantly differently from the control. Mechanistic genes do not necessarily undergo a large fold change; a small fold change in a mechanistic gene may yield much greater changes in downstream genes. Where Gene Ontology (GO) pathways are discussed, *Bos taurus* (*bta*) pathways are referenced. Where KEGG pathways are discussed, human (hsa) pathways are used instead of the bovine (bta) pathways due to completeness and annotation.

**Bronchoalveolar lavage samples.** Microarray analysis revealed that approximately 984 bovine genes were detected as differentially expressed (z-score $p < 0.0125$) in infected animals compared with pre-inoculation controls between 1-22 Days post-infection (**Table 4**). These genes encompassed 908 GO processes, with 43 highly-activated processes comprised of 274 genes (**Appendix A**) (processes downloaded from Gene Ontology Consortium Database; http://www.geneontology.org). One hundred twenty-five mechanistic genes were identified in this comparison (**Appendix B**). The overall expression profile of bronchoalveolar lavage fluid was one of upregulation, as upregulated genes outnumbered downregulated ones by a ratio of 2:1. Samples were also obtained on Day 29, but a low signal-to-noise ratio on Day 29 arrays prohibited the use of that data.

**Table 4. BAL gene expression**. Numbers of genes differentially expressed in bovine bronchoalveolar lavage fluid from 1-22 days post-infection with MmmSC.

| Time Point (Day) | # Genes upregulated | # Genes downregulated | Total # Genes differentially expressed |
|---|---|---|---|
| 1 | 201 | 41 | 252 |
| 8 | 201 | 87 | 288 |
| 15 | 149 | 58 | 207 |
| 22 | 163 | 74 | 237 |

The greatest number of transcriptional changes occurred in the *bta* GO groups chemokine activity, negative regulation of cell proliferation, viral genome regulation, defense response, sugar binding, and humoral immune response. All of these processes were highly active early in infection (Day 1) and decreased in activity later in infection.

Top-ranked KEGG pathways in BAL samples included cytokine-cytokine interaction (hsa04060), lectin-induced complement pathway (hsa99020), apoptosis (hsa04210), androgen and estrogen metabolism (hsa00150), Toll-like receptor signaling (hsa04620), antigen processing and presentation (hsa04612), and T-cell receptor signaling (hsa04660) (**Appendix C**). The cytokine-cytokine interaction pathway was upregulated. Genes CCL2, IL8, IL1A and IL1B all had moderate to high upregulation at all time points. These genes, plus IL8RB, were all found to be mechanistic; 81 of 235 genes in the pathway were assessed. Two of 9 genes of the lectin-induced complement pathway were measured and C2 was upregulated with moderate downregulation of C4A.

Both of these genes were found to be mechanistic. Twenty-seven of 56 genes of the apoptosis pathway were interrogated. This pathway was upregulated at Day 1, but Day 8 onward revealed down regulation of MYD88. Two genes were found to be mechanistic: IL1B and NFKBIA. None of 18 genes of the androgen and estrogen receptor pathway were assayed. Pathway intermediates HSD11B1 and HSD11B2 were downregulated at all time points, but CYP11B1 was strongly upregulated at all time points. Both CYP11B1 and HSD11B1 were identified as mechanistic genes. The Toll-like receptor signaling pathway was primarily upregulated at all time points with 38 of 65 pathway genes assayed. The exception to the upregulation was IL6, which was either downregulated or had no change from baseline at all time points. IL12A was downregulated on Day 22. Mechanistic genes in the TLR pathway included TLR3, NFKB1, NFKBIA, IL1B, IL6 and IL8. The antigen processing and presentation pathway had a pattern of slight upregulation early in infection, which changed to slight downregulation by Day 15. Eleven of the 18 TLR pathway genes were investigated. Mechanistic genes identified in this pathway were PSME1, TAPBP, Tap2 and PSME2. The T cell receptor signaling pathway was investigated by measuring 21 of 49 pathway genes, and the pattern was again one of upregulation at all time points. Four mechanistic genes were identified including PTPN6, IKBKG, NFKB1 and NFKBIA.

**Bronchial biopsy samples.** Microarray analysis revealed that approximately 3218 bovine genes were detected as differentially expressed (z-score $p < 0.0125$) in infected animals compared with pre-inoculation controls between 1-22 Days post-infection (**Table 5**). These genes encompassed 908 GO processes, with 49 highly-

activated processes comprised of 518 genes (**Appendix D**). Three hundred fifty-six mechanistic genes were identified in this comparison (**Appendix E**). The overall expression profile of bronchial biopsy samples was one of downregulation, with downregulated genes outnumbering upregulated ones by a ratio of 1.5:1 to 4:1 at time points from days 1-22.

**Table 5. Bronchial biopsy gene expression**. Numbers of genes differentially-expressed in bovine bronchial biopsies from 1-22 days post-infection with MmmSC.

| **Time Point (Day)** | **# Genes upregulated** | **# Genes downregulated** | **Total # Genes differentially expressed** |
|---|---|---|---|
| 1 | 591 | 2384 | 2975 |
| 8 | 951 | 1651 | 2602 |
| 15 | 1185 | 1227 | 2412 |
| 22 | 1023 | 2173 | 3196 |

The most transcriptional changes occurred in *bta* GO processes extracellular matrix structural constituent, protein transporter activity, protein secretion, collagen catabolism and metalloendopeptidase activity. The extracellular matrix structural constituent process was most active starting at Day 8, while the protein processes were most active on Day 1. Collagen catabolism increased slightly over time, while metalloendopeptidase activity was relatively constant post-infection.

In bronchial biopsy samples, the top five ranked KEGG pathways were oxidative phosphorylation (hsa00190), lectin-induced complement pathway (hsa99020), natural

killer cell mediated cytotoxicity (hsa04650), glutathione metabolism (hsa00480), and prostaglandin and leukotriene metabolism (hsa00590) (**Appendix F**). Seventy-five of 152 oxidative phosphorylation genes were assayed, revealing prominent downregulation with the exception being COX7B. No mechanistic genes were identified from this pathway. Two of 9 lectin-induced complement pathway genes were assayed, disclosing downregulation of C4A. C2 was downregulated in early infection but was upregulated from Day 15 onward, and both genes were found to be mechanistic. Thirty-two of 73 natural killer cell mediated cytotoxicity pathway genes were assayed. The pattern was mostly one of downregulation, with only genes RAC1, RRAS, FYN and TNFRSF6 having upregulation at any time point. Sixteen mechanistic genes were discovered, including MAPK1, FYN, several subunits of PIK3, ICAM1, ITGAL, TNFRSF6, RAC1 and SYK.

**Nasal cytology samples.** Microarray analysis revealed that approximately 2043 bovine genes were detected as differentially expressed (z-score $p < 0.0125$) in infected animals compared with pre-inoculation controls between 1-22 days post-infection (**Table 6**). These genes encompassed 908 GO processes, with 45 highly-activated processes comprised of 329 genes (**Appendix G**). One hundred seventy-six mechanistic genes were identified in this comparison (**Appendix H**). In cytology samples, the gene expression profile varied widely by time point. Initially upregulation was dominant, but downregulated genes were more prevalent at Day 8. The profile returned to one of upregulation from Days 15-22.

**Table 6. Nasal cytology gene expression**. Numbers of genes differentially expressed in bovine nasal cytology samples from 1-22 days post-infection with MmmSC.

| Time Point (Day) | # Genes upregulated | # Genes downregulated | Total # Genes differentially expressed |
|---|---|---|---|
| 1 | 827 | 252 | 1079 |
| 8 | 493 | 764 | 1257 |
| 15 | 525 | 414 | 939 |
| 22 | 467 | 222 | 689 |

The *bta* GO processes demonstrating the largest number of transcriptional changes were T-cell receptor complex, mitosis, myogenesis, microtubule associated complex and oxygen and reactive oxygen species metabolism. Most of these processes were relatively constant over time post-infection. The T-cell receptor complex process had high activity on Day 1 followed by a decrease on Day 8 and a gradual increase thereafter.

The top five KEGG pathways for cytology samples included glycerolipid metabolism (hsa00561), oxidative phosphorylation (hsa00190), CD-40L signaling pathway (hsa99110), TGF-beta signaling pathway (hsa04350), and epithelial cell signaling in *Helicobacter pylori* infection (hsa05120)(**Appendix I**). Sixteen of 30 glycerolipid metabolism pathway genes were analyzed which had a predominant pattern of downregulation at all time points. The exception was gene YWHAZ, which was upregulated throughout. Five mechanistic genes were identified: PCAF, PLA2G1B, PPAP2C, YWHAZ and AGPAT1. Seventy-five of 152 genes of the oxidative

phosphorylation pathway were analyzed and were primarily downregulated, but in this tissue several of the COX subunits were upregulated. Three mechanistic genes were identified: ATP5A1, ATP5F1 and ATP5G2. Seven of thirteen genes in the CD-40L signaling pathway were investigated, revealing that the pathway was upregulated on Day 1 but downregulated later in infection; however, gene CHUK was consistently upregulated and gene IKBKB was consistently downregulated. Three mechanistic genes were identified: TNFRSF5, IKBKB and CHUK. Twenty-five of 43 genes of the TGFβ signaling pathway were evaluated. The pathway appeared to be upregulated at Day 1, but many genes were downregulated thereafter; genes ID1 and ID3 remained upregulated at all time points. Eight mechanistic genes were identified: SMAD1, SMAD2, SMAD4, ID1, ID3, BMP4, AMH and BMPR1A. Eleven of 25 genes of the epithelial cell signaling in *H. pylori* infection pathway were assayed. The pathway was upregulated at Day 1, with genes CHUK and CCL5 remaining upregulated. Genes NFKBIA and NFKB1 were downregulated from Day 8 onward. Three mechanistic genes were found: CCL5, CHUK and NFKB1.

**Blood samples.** Microarray analysis revealed that approximately 924 bovine genes were detected as differentially expressed (z-score $p < 0.0125$) in infected animals compared with pre-inoculation controls between 1-22 days post-infection (**Table 7**). These genes encompassed 908 GO processes, with 49 highly-activated processes comprised of 327 genes (**Appendix J**). One hundred five mechanistic genes were identified in this comparison (**Appendix K**). The gene expression profile was

predominantly one of mild upregulation early in infection (Days 1-15), but downregulation predominated on Day 22.

**Table 7. Blood gene expression**. Numbers of genes differentially-expressed in bovine blood from 1-22 days post-infection with MmmSC.

| Time Point (Day) | # Genes upregulated | # Genes downregulated | Total # Genes differentially expressed |
|---|---|---|---|
| 1 | 180 | 95 | 275 |
| 8 | 148 | 127 | 275 |
| 15 | 179 | 81 | 260 |
| 22 | 109 | 454 | 563 |

The *bta* GO processes having the greatest change in gene expression were G-protein signaling, coupled to cAMP nucleotide second messenger, endonuclease activity, Ras protein signal transduction, nuclease activity and ATP-dependent RNA helicase activity. The first process was relatively constant over time post-infection, while the others were highest on Day 1 and decreased slightly thereafter.

The top-scoring KEGG pathways for blood included heparan sulfate biosynthesis (hsa00534), chondroitin/heparan sulfate biosynthesis (hsa00532), ethylbenzene degradation (hsa00642), arginine and proline metabolism (hsa00330), and tyrosine metabolism (hsa00380) (**Appendix L**). Three of 33 genes of the heparan sulfate pathway were evaluated and one gene, GLCE, was both strongly upregulated and mechanistic. Three of 8 genes of the chondroitin/heparan sulfate biosynthesis pathway were measured,

revealing upregulation in EXT2 and downregulation in XYLT2 and B3Gat3 at all time points, but only B3Gat3 was determined to be mechanistic. Three of 38 genes of the ethylbenzene degradation pathway were evaluated, with downregulation occurring in all genes. The gene PCAF was determined to be mechanistic. Twenty-seven of 40 genes in the arginine and proline metabolism pathway were assayed, disclosing a pattern of mixed regulation, with many genes switching from an upregulated to downregulated state or vice versa during the course of infection. The single gene identified as mechanistic was ARG2. Twenty-four of 190 genes of the tyrosine metabolism pathway were assayed, revealing increasing upregulation over time. Seven genes, TH, HEMK1, ADC3, TYR, Tat, DCT and TYRP1, were found to be mechanistic.

**Comparison of microarray and RT-PCR results.** Sixteen genes were selected for real-time PCR comparison with microarray results (**Table 8**)(**Figure 26**). Genes were randomly selected from those determined to be mechanistic via microarray data analysis, so not all genes selected were differentially expressed in every tissue type or at every time point analyzed. Results were considered to be in agreement if the direction of fold change determined via microarray (up- or downregulation) agreed with direction of fold change via real-time PCR. Agreement between microarray results and RT-PCR results over the sixteen genes analyzed was 88% for bronchoalveolar lavage fluid, 92% for bronchial biopsy, 90% for nasal cytology and 89% for blood. Agreement within time points of a gene was very consistent; disagreement typically included all time points of a given gene rather than individual time points of several genes.

**Table 8. Primers for real-time PCR analysis of bovine genes**

| GenBank Accession # | Gene Symbol | Gene Product | Forward Primer (5'-3') | Reverse Primer (5'-3') |
|---|---|---|---|---|
| NM_174093 | IL1B | Interleukin 1β | CTTGGGTATCAAGGACAAGAATCTA | TAGGGTACAGGACAGACTCAAATTC |
| NM_001012671 | STAT3 | Signal transducer and activator of transcription 3 | GGATAACCTCATTAGCAGAATCTCA | TAAGTTTCTAAACAGCTCCACGATT |
| NM_174091 | IL18 | Interleukin 18 | CTGTGAGAACAAAATTGTTTCCTTT | TTCTGGTTTTGAACAGTGAACATTA |
| NM_181003 | Aqp4 | Aquaporin 4 | AATAGCTGATAATCAAGGGCTTTCT | GTGTGATGTCTCTCTGGAACCTTAT |
| NM_173877 | F2 | Complement factor 2 | GATGGAAAATATGGCTTCTACACAC | AGGAACAAACCACAAAAATAATTCA |
| NM_174062 | FTH1 | Ferritin heavy chain 1 | TCTTCCTTCAGGATATCAAGAAACC | GAGTCTCAATGAAATCACACAGATG |
| NM_174101 | LGMN | legumin | GTATGCTTTGAGACATTTGTACGTG | TCTCTGATTAGCACACATTGTAAGG |
| NM_175793 | MAPK1 | mitogen-activated protein kinase 1 | GGCTTGGCCCGTGTTG | GGAAGATGGGCCTGTTGGA |
| NM_173925 | IL8 | Interleukin 8 | TGCTTTTTTGTTTTCGGTTTTTG | AACAGGCACTCGGGAATCCT |
| NM_173923 | IL6 | Interleukin 6 | AAACCGAAGCTCTCATTAAGCG | TGGAAGCATCCGTCCTTTTC |
| XM_001250668 | CALM2 | Calmodulin 2 (predicted) | CAAAGGAATTGGGAACTGTAATGA | TCATGTCCTGTAACTCTGCTTCTGT |
| BC_105484 | NFKBIA | NFKB inhibitor activator | TCCTGCAGGCCACCAACTAC | GCCATGAATAGAGGCCAAGTG |
| NM_174348 | ICAM1 | Intercellular adhesion molecule 1 | TCTTGCCGCTGGGAACTG | GGCCGAGGTGTTCTGGAA |
| NM_198221 | ITGAL | Integrin alpha L | GGAAGCCACCGACGAACA | TCCCAATGATGTAGCGGATGA |
| BC_114192 | TGFB | Transforming growth factor beta | CGCAGAGCGGCCAAAG | AAGACGAGCAATTTCATCACAAATAG |
| NM_173893 | B2M | β2-microglobulin | ATGGAAAGCCAAATTACCTGAA | GGGTTGTTCCAAAGTAACGTGT |

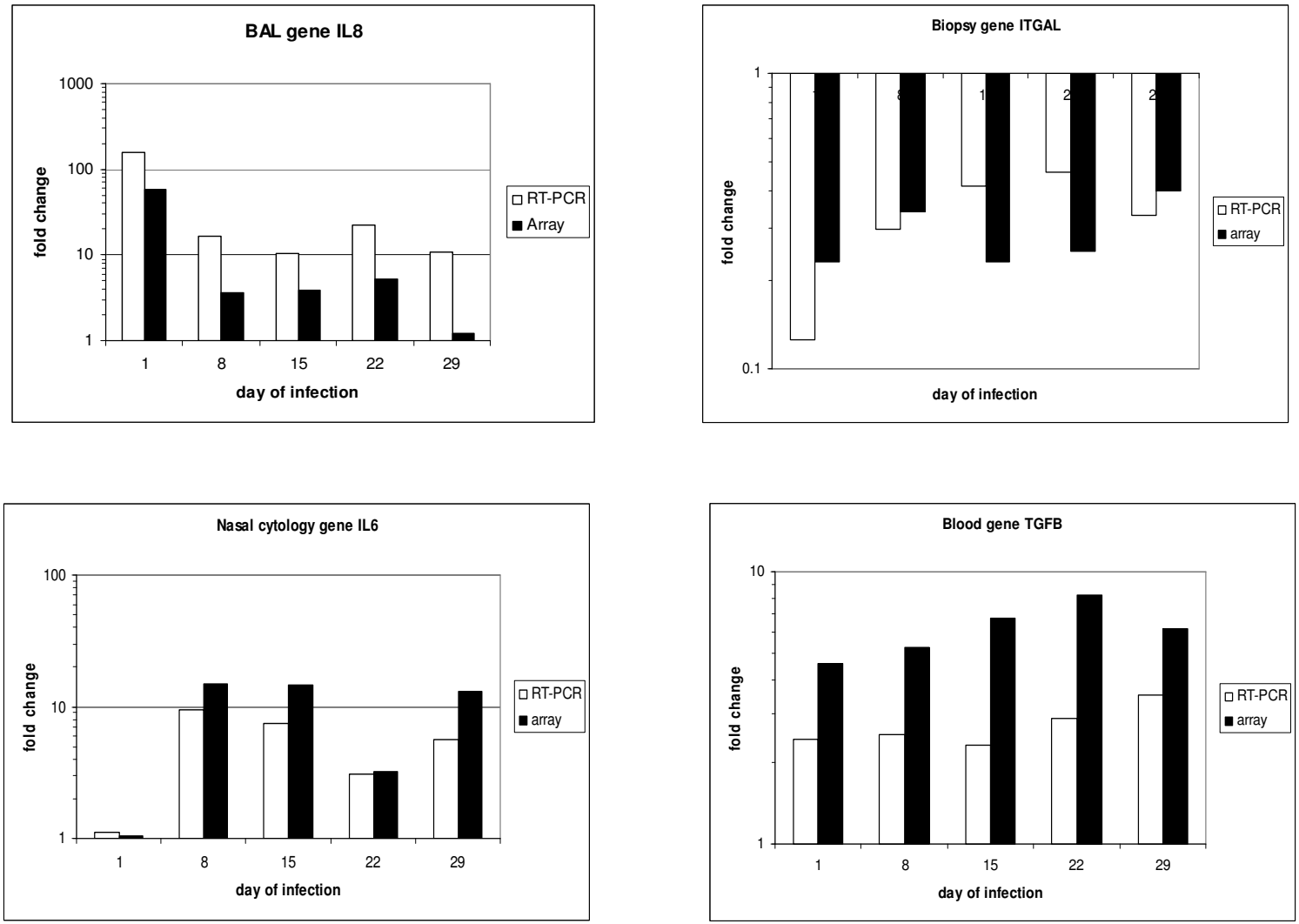



**Figure 27. Microarray-PCR comparison**. Representative graphs comparing microarray and real-time PCR results for selected genes and tissues. One representative gene is shown for each tissue type assayed.

## DISCUSSION

The mechanisms by which MmmSC evades or circumvents the host immune response are poorly understood, as are the details of how the host responds to infection. Most host response data for mycoplasmas focuses on the human or murine pathogens M. *pneumoniae* or *M. arthriditis*, which are phylogenetically distant from the mycoides cluster[40]. It was a goal of this study to generate a global gene expression profile of cattle infected with MmmSC in an attempt to discover genes or pathways important in the molecular pathogenesis of infection.

Involvement of the immune system is necessary for any host to overcome a pathogen. Naïve hosts must first rely on the capabilities of the innate immune system, including granulocytes, monocytes/macrophages, natural killer cells, complement, and acute phase proteins. The bronchoalveolar lavage samples obtained in this study should contain primarily macrophages, with variable numbers of neutrophils dependent on the state of infection[110], although BAL cytology was not done during this study due to logistics issues.

Investigation of the bovine host response to MmmSC infection had an early enhancement of the transcriptional profile followed by a gradual decline. Inflammatory processes were strongly represented. Microarray analysis of BAL samples revealed upregulation in a number of innate immune system genes in the first day following infection. Macrophage-associated genes exhibiting early (Day 1) upregulation included CSF1, which stimulates differentiation of mononuclear phagocytes. The proinflammatory neutrophil chemoattractant interleukin 8 (IL8) exhibited a dramatic

Day 1 increase (58-fold baseline) and the IL8 receptor was upregulated. IL1A and IL1B were also significantly increased in expression. The natural killer cell attractant IL12 was moderately upregulated. The gene CCR1, a macrophage stimulatory receptor, and its ligand CCL20 had strong early increases in transcription. Monocyte chemotactic factors CCL2 and CCL8 also were upregulated early in infection, with CCL2 exhibiting a 34-fold increase over baseline levels on Day 1. Multiple components of major histocompatibility class II (MHCII) complex molecules were increased in expression starting at Day 1 post-infection, potentially indicating immune system response to exogenous MmmSC antigen.

The adhesion molecule ICAM1 was increased in expression on Day 1, as was L-selectin (SELL). A gene similar to CD209, Bt.9532, was upregulated. CD209, which is expressed on dendritic cells, is a C-type lectin that stabilizes T cell receptor interaction[111]. Lysosomal genes also demonstrated increases in expression, including LAMP1, the lysosomal cysteine protease CTSB, a cathepsin A-like transcript, and lysosomal α-mannosidase (MAN2B1). A number of innate immune system genes normally associated with antiviral response were upregulated, including OAS1, MX2, and UBE1L. The antiviral gene Isg15, a ubiquitin homolog that is believed to influence dendritic cell migration[112], was significantly increased in expression through Day 8, as was the putative interferon-induced transmembrane protein Bt.64826. A transcript (Bt.33922) resembling the antiviral gene radical S-adenosyl methionine domain containing 2 (viperin) was also strongly upregulated on Days 1 and 8; viperin is known

to be induced by human cytomegalovirus infection, but is also induced by LPS and interferons[113,114].

There was also a large increase in expression of a gene resembling the eotaxin receptor (Bt.28967), which demonstrated a 46-fold increase over baseline levels on Day 1 and remained at or above 10-fold above baseline throughout the experiment. However, eotaxin expression was not changed. The gene NCR3, known to mediate dendritic cell/NK cell crosstalk as well as NK cell cytotoxicity[115], was moderately upregulated. The NK-cell and regulatory T cell-associated gene NKG7[116-118] also demonstrated significantly increased expression throughout infection. Natural killer cells are known to be activated by mycoplasma infection[119,120], but their precise role in clearance of infection has not been determined.

Other differentially-regulated genes associated with the innate immune system included complement factor 2 (C2), which was upregulated while C3 had moderate downregulation. This could indicate preferential activation of the mannose-binding lectin complement pathway over the alternative pathway in early infection. This may be supported by clinical evidence suggesting that mannose-binding lectin deficiency confers susceptibility to mycoplasma infections in humans[121]. Slightly later in the experiment (Day 8), classical pathway complement components C1qa and C1qb were increased in expression, indicating that antibody-antigen complexes were likely present by this time. Further supporting the presence of immune complexes was an upregulation of the gene Bt.28230, similar to complement component H, on Days 8 and 15. Component H traffics immune complexes from the site of formation to phagocytic cells

and limits the deposition of immune complexes in tissues[122]. The anticoagulant gene ANXA8 was downregulated while fibroleukin (FGL2) was upregulated, suggesting a procoagulant state. FGL2 has also been suggested to downregulate T cell proliferation and dendritic cell maturation[123]. In contrast to the procoagulant genes, the platelet adhesion factor CD36 was moderately downregulated.

Additional innate immune components including the adhesion factor CD164, NOD2-like CARD15, the CCR5-binding ligand CCL3LI, and the antioxidant gene Sod2 demonstrated moderately increased expression in early infection. The gene Bt.643, whose product is similar to the neutrophil-secreted macrophage chemoattractant macrophage inflammatory protein (MIP), was strongly upregulated on Day 8 but not Day 1. This corresponds to the observed increase in pulmonary neutrophils over the course of CBPP.

The bovine gene Bt.6915, which is similar to the human hemoglobin scavenger CD163, exhibited up to 30-fold baseline expression. CD163 is produced in both membrane-bound and soluble forms by cells of the monocyte/macrophage lineage and the soluble form is shed in large amounts by activated macrophages[124]. Soluble CD163 is believed to be an anti-inflammatory mechanism, as scavenging of free hemoglobin decreases oxidative damage[125]. Shedding of soluble CD163 by human monocytes may be induced by activation of Toll-like receptors TLR2, TLR4 and TLR5[126-128]. Interestingly, of these three Toll-like receptors only TLR4 had increased expression in BAL samples from the current experiment. TLR4 recognizes the lipopolysaccharide (LPS)/lipopolysaccharide binding protein (LBP)/CD14 complex, which should only be

available in the presence of Gram-negative pathogens. MmmSC, like all mycoplasmas, lacks a cell wall and does not elaborate LPS, although some mycoplasmas have been reported to activate the lipoprotein pattern recognition receptor TLR2[129,130]. It is unclear from these data whether the increase in CD163 expression is due to TLR4 activation by an unrecognized Gram-negative pathogen present in the experimental animals or due to TLR2 activation without increased TLR2 expression. The Bt.6915/CD163-like transcript was strongly upregulated (16- to 42-fold) throughout the course of the experiment.

The adaptive immune system is also involved in the response to MmmSC infection although, predictably, more so later in the course of disease. Early indications of adaptive immunity are seen in the Day 1 expression of IL12B, which induces lymphocyte differentiation into the T-helper subset 1 ($T_H1$) phenotype. The CD80 gene, necessary for CD28 co-stimulation of T cells, also had increased expression between Days 1-15. Several components of the T cell receptor complex CD3 were upregulated by Day 8, and a gene (Bt.26847) similar to linker for activation of T cells (LAT) was also moderately upregulated. The LAT gene is expressed following activation of the T cell receptor (TCR) and recruits proteins into complexes near the site of the TCR. Several genes associated with heat shock proteins were upregulated on Day 8. While heat shock proteins are considered part of the innate immune system, when complexed with antigen and presented to dendritic cells they are known to strongly activate $T_H1$ cells[131-133]. Transcripts of the chaperonin Cct8, known to be upregulated by T cell

activation[134], were also increased on Day 8. Expression of Bt.28015, the provisional bovine Fcγ2 receptor, was also moderately increased through Day 15.

The T cell pathways were not exclusively activated by MmmSC, but involvement of the humoral immune system in BAL samples appeared less prominent. The B cell growth-associated receptor CXCR4 (also known as stromal cell derived factor (SDF) 1α/β receptor) was upregulated, increasing in expression to Day 22, although SDF1 expression was only slightly above baseline. The gene for the immunoglobulin j-chain remained unchanged until Day 22, when it increased 13-fold. The IL13 receptor subunit α-1 chain precursor expression was upregulated on Day 8, although IL13 was slightly downregulated at all time points. IL13's effects include B cell growth and differentiation, inhibition of the $T_H1$ response and inhibition of macrophage inflammatory mediator production. This expression pattern could suggest a need for continued activation of the $T_H1$ response while maintaining maximal sensitivity for the humoral response. Also appearing to diminish the contribution of the humoral immune system were decreases in transcription of the polymeric immunoglobulin receptor (pIgr), whose primary role is to transport secretory IgA through epithelial surfaces. The overall appearance of the adaptive immune system during MmmSC infection appears to be one of primarily $T_H1$ activation with a minimal $T_H2$/B cell contribution. However, the lack of transcripts from either $T_H1$ or $T_H2$ cell secreted products makes assessment tentative at best. The lack of T lymphocyte transcripts may again be due to the predominantly macrophage/neutrophil cell type harvested by BAL. In normal animals there are 2 to 4.5% lymphocytes in BAL fluid[110], but those

lymphocytes are reported to be mostly memory and $CD8^+$ cells[135,136]. These studies suggest that fewer than 0.4% of BAL cells in normal cattle are expected to be $CD4^+$ T cells; this may be a partial explanation of the lack of detection of RNA transcripts in BAL fluid from either subpopulation of $CD4^+$ cells. No studies have specifically investigated alveolar lymphocyte subpopulations of cattle with bacterial respiratory disease, but an investigation of feedlot calves with naturally-occurring respiratory disease did not reveal differences in total lymphocyte percentage between affected and control calves[137,138]. It would have been useful to directly assess soluble T helper subset cytokines in BAL fluid.

The immune system is not entirely upregulated following infection with MmmSC. Increased expression of SOCS3 at Day 1 indicates that there is some restriction of the proinflammatory cascade. A degree of restraint is also indicated by the increased expression of TNFA1P6 (TSG6), which inhibits neutrophil migration and inflammation[139,140]. The matrix metalloproteinase (MMP) inhibitor TIMP1 was increased in expression through Day 8, although MMPs were only slightly increased in expression in BAL fluid. Matrix metalloproteinases degrade connective tissue matrix, and MMP2 and MMP9 have been identified in mycoplasmal disease[141,142]. Metalloproteinase inhibitors have been investigated as mediators of disease[143]; in a model of Johne's disease, increased expression of TIMP1 was associated with persistence of the bacteria in cultured cells[144], possibly suggesting a role for this gene in failure of disease clearance. Vanin-1, a gene associated with granuloma formation and leukocyte transendothelial migration[145-147], was downregulated in early infection, and

IL1R2, an inhibitor of IL1, was upregulated at Days 1 and 8. The negative regulator of T cell activation Vsig4[148] was also increased in expression through Day 8. A gene similar to the mitogen-activated protein kinase (MAPK)-related nemo-like kinase was strongly upregulated in early infection. Nemo-like kinase (NLK) inhibits beta-catenin lymphoid enhancer factor/T cell factor-mediated transcription[149,150], and has also been implicated in suppression of nuclear factor kappa B (NFκB)-mediated transcription[151]. Nemo-like kinase is reported to be activated by STAT3 and IL6[152], but neither were significantly differentially regulated in this sample type. This suggests that an alternate activation mechanism for NLK may be present, or that this bovine NLK homologue may be activated via a different pathway than human or murine NLK.

A number of other intriguing avenues are suggested by the data. For example, the gene for connexin 43 is dramatically upregulated early in infection, reaching 148-fold of baseline by Day 8. Connexin 43, a component of the gap junction, has been implicated in the spread of $Ca^{2+}$-mediated acute lung injury and is also known to be induced by LPS[153,154]. The presence of increased expression by several LPS-responsive genes in BAL samples suggests either that TLR4 downstream pathways are being activated by other receptors (possibly TLR2 or TLR6) or the presence of an unidentified Gram-negative organism in the experimental animals. Although two of the animals had evidence of resolved anteroventral pneumonia, characteristic Gram-negative organisms or active lesions were not identified at necropsy.

The gene aquaporin 4 (Aqp4) was moderately downregulated throughout infection. Aquaporins are integral membrane proteins that permit water transport along

osmotic gradients, and Aqp4 is known to be present in pulmonary epithelial cells. Aquaporins have been deemed unimportant in pulmonary water balance but have been implicated in the development of cerebral edema[155,156], although little work has been done in models of lung injury[157]. The decreased expression of Aqp4 in this experiment may potentially indicate one mechanism involved in the formation of the characteristic pulmonary interstitial edema seen in CBPP.

Transcriptional responses were also frequently upregulated in early infection, as were antiapoptotic mechanisms. Mail (NFKBIZ), an IL1-induced modulatory regulator of NFκB-mediated transcription[158-160], was profoundly upregulated in early infection (104-fold). Helix-loop-helix transcription factor BHLHB2 as well as both transcriptional mediators NFKB1 and NFKBIA were highly activated, and the NFκB activator and antiapoptotic gene osteopontin similarly had dramatic increases in expression. Additional antiapoptosis genes having increased expression were GADD45B and BCL2A1. The pro-apoptotic gene Peg3 was downregulated throughout the experiment, but CASP4 was upregulated. The overall trend was one of antiapoptosis, which was supported by the substantial increases in transcripts of multiple transcription factors and ribosomal proteins.

The overall picture seen in bronchoalveolar lavage samples of MmmSC-infected cattle following infection is one of significant activation of the innate immune system, antiapoptosis, and large-scale transcriptional activation. Multiple cytokine and chemokine production by macrophages recruit immune cells of both the innate and adaptive arms to the affected lung. This activation continues at a lower level through

Day 8, and most innate immune system genes return to near-baseline levels by Day 15. Transcriptional activity also diminishes by Day 15, and most antiapoptosis genes trend toward baseline levels of transcription. This gradual decrease in transcription mirrors the clinical picture in which infected animals that survived acute disease showed resolution of clinical signs.

Results from this study are not in complete agreement with previous research on CBPP. For example, TNFα was not significantly differentially regulated in bronchoalveolar lavage samples in this study, despite previous *in vitro* evidence demonstrating induction of macrophage TNFα production by virulent MmmSC[64]. However, TNF receptors were moderately upregulated in the current study. Our experiment did demonstrate significant increases in expression of IL1A and IL1B, which are also produced by TNFα-producing macrophages, but did not find evidence of increased macrophage IL6 production in BAL samples. The study mentioned above also demonstrated that pathogenic strains of MmmSC fail to induce procoagulant activity[64], while the results from the current study demonstrate a procoagulant state following infection with MmmSC. This disagreement may indicate a difference between *in vitro* versus *in vivo* results or strain differences between the mycoplasmas. An *ex vivo* experiment generated evidence that IFNγ-producing $CD4^+$ T cells were necessary for recovery from CBPP[65]. The current investigation, again, did not provide evidence of differential regulation of IFNγ in bronchoalveolar lavage samples. In addition, the current study revealed moderate downregulation of IL18, also known as interferon gamma inducing factor. As the previous study evaluated IFNγ production using a

lymphoproliferation assay on peripheral blood mononuclear cells, reasons for the differing results could involve both variances between PMBC and alveolar macrophages and/or altered behavior of the cells outside the normal bovine environment. Results may also differ because this aspect of the current study used pooled samples that did not take into account which animals survived and which did not. A follow-on study to the one mentioned above suggested that live MmmSC could secrete a substance that induced apoptosis in both lymphocytes and granulocytes[66]. Although this substance has not been further defined, the study authors suggest that hydrogen peroxide should be considered for the role. Our results demonstrate increased expression of two superoxide dismutase genes but no glutathione peroxidase genes, suggesting that hydrogen peroxide production was not prominent in BAL samples.

*In vivo* studies of the immune response have demonstrated that cell-mediated immunity is important in the resolution of clinical signs[65]. Additionally, MmmSC appears to be able to inhibit concanavalin A-induced blastogenesis and IFNγ production by PBMC and lymphocytes[67], further intimating that cell-mediated immunity may be necessary for control of infection. Our study failed to demonstrate mRNA in BAL associated with a cell-mediated response such as IFNγ, although gene expression products hinted at both a $T_H1$ response and a humoral response. Contrary to the observations championing cell-mediated immunity is one study that demonstrated that passive immunity to CBPP could be transferred via serum[68]. Investigations of the humoral immune response to MmmSC infection has documented a potent IgA response local to infection and a weaker systemic IgG response[53], and that the response is stronger

in animals infected with more virulent strains of MmmSC. A different study suggested that increased IgA production was correlated with less severe disease, while other immunoglobulin isotypes were similar between animals with acute disease and those with subacute or chronic disease[69]. Multiple studies have suggested that the host response is in part responsible for the severity of lesions observed in CBPP, but the extent of this contribution is undetermined[31,70,71]. Our investigations did not demonstrate increased expression of IgA or IgG, although several receptors for IgG were upregulated in early infection. The Ig joining chain was prominently upregulated, however, which may be indirect evidence of increased IgA levels. As was previously stated, the BAL cell population would not be expected to contain large numbers of B lymphocytes, which may explain the absence of detectable Ig transcripts. This study was not able to directly sample lymphoid tissue during infection; such tissue would have been the desired sample for determination of immunoglobulin gene expression.

Investigation of specific pathways involved in the bovine response to MmmSC infection may shed light on the mechanisms by which the host responds to infection. For example, the current study found a significant increase in the expression of interleukin 8 and interleukin 1B in bronchoalveolar lavage samples. Interleukin 8 is a CXCL cytokine secreted by cells of the monocyte/macrophage lineage whose primary effect is that of polymorphonuclear leukocyte chemotaxis to mucosal surfaces. IL8 is known to be induced by lipopolysaccharide; complement factor Xa, thrombin and fibrin can also activate endothelial cells, eliciting the synthesis of IL6 and/or IL8. Little is known about the involvement of IL8 in mycoplasmal infections; a single *in vitro* study

of swine coinfection with *M. hyopneumoniae* and Porcine Respiratory and Reproductive Syndrome Virus identified increases in IL8 mRNA in pulmonary alveolar macrophages as well as soluble IL8 in culture supernatant, but this was true also for cells infected with either organism alone[161]. A bovine *in vitro* study suggests that IL8 is a mediator of LPS-induced endothelial injury in models of *Mannheimia haemolytica* infection[162]. Given that mycoplasmas do not elaborate LPS, another component of MmmSC may be activating TLR4 normally activated by LPS or activating downstream pathways by other receptors. It is known that diacylated lipopeptides present in some mycoplasmas are capable of binding to LPS-binding protein (LBP)[163,164] and activating cells via TLR2[130]; it appears that MmmSC may be added to that list. Some mycoplasmas, including *M. arthritidis* and *M. fermentans*, are known to elaborate superantigens such as macrophage activator lipoprotein-2 (MALP-2) that induce a shock-like syndrome identical to that induced by LPS[165]. Neither MALP-2 nor any other superantigen has been identified as being produced by organisms in the mycoides cluster. A study on the mechanisms of IL8 induction in human cells suggested that the extracellular signal-regulated kinase/ mitogen-activated protein kinase (ERK/MAPK) pathways were activated by mycoplasmal lysate[166], although the specific mycoplasmal component was not identified. In the current study, the cytokine-cytokine receptor pathway, the MAPK signaling pathway and the Toll-like receptor signaling pathway were all ranked in the top 10 for bronchoalveolar lavage samples, supporting the importance of these pathway components in disease pathogenesis.

**Bronchial biopsy samples.** The samples collected of bronchial tissue were expected to contain primarily ciliated bronchial epithelial cells. The samples were taken with a 2.5mm endoscopic biopsy forcep and were found to be too small for standard histologic evaluation due to crush artifact; actual cell population could not be determined. Innate immune system activation was dramatically different from that seen in bronchoalveolar tissue. There was significant downregulation of most genes seen to be upregulated in BAL samples. The exceptions included IL1A/B, which remained significantly upregulated, and granulocyte-monocyte colony stimulating factor (CSF2), which was moderately increased in expression. Interleukin 6 (IL6) was significantly increased in transcription over baseline, which was not seen in BAL samples. This suggests that the epithelial tissue macrophages respond differently than BAL macrophages to MmmSC. The monocyte chemoattractant CCL2 had increased transcription on Days 15 and 22, but not early in infection. The T cell receptor CXCR3 and ligand CCL5 (RANTES) both had increased expression, supporting the presence of both T cells and macrophages in biopsy samples. Adhesion factor CD164 was upregulated, as were antiviral-associated genes OAS3 and MAN2B1. Coagulation cascade components Factor 9 and Factor 11 were upregulated, indicating a procoagulant state and activation of the intrinsic coagulation cascade. This supports the report of MmmSC-induced procoagulant activity[64], and may also reflect an MmmSC-potentiated response to tissue trauma caused by harvesting the biopsy. Increased expression of complement component 5 suggests the construction of complement membrane attack complexes as well as increased chemotaxis of leukocytes by C5a. The complement

component H-like gene Bt.28230 was upregulated throughout the experiment, likely indicating the formation of immune complexes within epithelial tissue. Most heat shock proteins were decreased in transcription, but one (DNAJC3) was upregulated.

The adaptive immune response in bronchial epithelial samples was again different from that in BAL fluid. The IL2 receptor was moderately downregulated throughout the sampling period. In addition, IL10 demonstrated moderate to large increases in transcription. Interleukin 4 was not differentially expressed, but its receptor was upregulated. These changes, plus the lack of increased expression of any $T_H1$-related transcripts, suggest that the bronchial epithelium is biased toward a $T_H2$-like response. However, we did not detect significant immunoglobulin heavy or light chain transcripts. Intriguingly, the immunoglobulin components Igj and pIgr were profoundly downregulated; this might be thought an unexpected finding given that secretory immunoglobulin should be present at epithelial surfaces. An alternative hypothesis might be that infection with MmmSC downregulates IgA production, allowing increased microbial persistence. Studies on *M. pneumoniae*[167,168] and *M. pulmonis*[169] have suggested that local immunity determines resistance to infection while systemic immunity is responsible for the pulmonary and systemic reactions, so downregulation of secreted IgA could be a useful means for MmmSC to establish a foothold in the lung. No studies have explored this possibility in mycoplasmas. However, the lack of Ig-related gene expression may be again explained by the possibility that we failed to capture epithelial lymphocytes and instead retrieved only non-Ig-secreting epithelial

cells. Secreted antibody may have been present but we did not investigate its occurrence.

One of the top-ranked pathways for differential gene expression in bronchial biopsy samples was the natural killer cell-mediated cytotoxicity pathway. In comparison to BAL samples, this pathway was almost entirely downregulated, with 25 of 27 evaluated transcripts demonstrating decreases in expression. As a number of mycoplasmas have been demonstrated to activate NK cells[119,120], the most likely explanation for the apparent inactivation of the NK cell pathway may again be the lack of effector cells in the harvested population. Alternatively, MmmSC may induce downregulation of NK cells to permit its own proliferation. No reports exist of this phenomenon in mycoplasmas. Pathways related to cell metabolism, while still downregulated, were less so than many of the immune system pathways. Once again, this is probably a reflection of the cell population acquired. It would be interesting to develop a method of harvesting bronchus-associated lymphoid tissue (BALT) periodically during infection to observe the gene expression profiles therein, but the endoscopic sampling method used in this experiment did not allow such discrimination of tissues.

**Nasal cytology samples.** Nasal brushings were expected to contain primarily squamous epithelial cells due to the superficial nature of the acquisition method and the location swabbed (level of the medial canthus of the eye). The construction of the sampling brush did not favor swabbing of pharyngeal lymphoid tissue, as the brush could not be angled after insertion into the nostril and the swabbing was done blindly. In

nasal cytology tissue the gene expression profile was quite different from that found in the lower respiratory tract, either BAL or bronchial biopsy. Most differentially-regulated genes were not evident until Day 8. Innate immune system involvement included evidence of NK cell activation via increased expression of NKG7, but the CXCR6 T cell/NK cell receptor was dramatically downregulated on Day 1 and moderately downregulated thereafter. The CCL5 ligand was increased on Day 1, but its receptors were not. The antiviral genes seen in BAL samples were not differentially regulated in nasal epithelial cells with the exception of UBE1L, which was highly expressed on Day 1 only. IL8 transcripts were increased on Day 1 only. CXCL6 (GCP2) was strongly downregulated on Day 8, and other neutrophil markers were not differentially regulated. This evidence suggests that neutrophils are not strongly recruited to nasal epithelium during MmmSC infection. Such evidence supports the reports that nasal swabs are an inconsistent source of the mycoplasma[81,170] and nasal epithelium is not a prominent site of infection. These findings are further supported by nasal swabs collected during this experiment, in which mycoplasma was isolated from only approximately 40% of the samples (data not shown).

Interleukin 1A in nasal epithelium was increased from Day 15 onward, while IL1B was increased only on Day 1. IL6 was highly upregulated from Day 8 onward. None of the cytokines associated with a $T_H1$ response (IFNγ, IL2, CSF2) were differentially regulated in nasal swab tissue. $T_H2$ cytokines IL3, IL5 and IL10 were also not differentially regulated, but IL4 had significant increases in expression starting on Day 8. T cell receptor CD3 components were increased in expression, again

predominantly from Day 8 onward. Complement components, with the exception of C5 and the factor H-like transcript (Bt.28230) were decreased in expression. This effect was most prominent on Day 1. C5 was moderately upregulated and Bt.28230 was highly upregulated throughout infection. Although the nasal epithelium is not a significant site of CBPP lesions, the intermittent MmmSC shedding by infected animals with the detection of Bt.28230 transcripts supports the implied presence of immune complexes in the tissue. Again, the Ig portion of the immune complex was apparently not produced by the cells that were sampled. The Ig joining chain and polymeric Ig receptor transcripts were also highly downregulated in nasal epithelial swabs. Minimal J-chain expression is expected since this component should be supplied by the Ig-synthesizing B lymphocyte. Secretory component and pIgr are manufactured by secretory epithelial cells, but it is unknown whether we retrieved any of these cells during sampling.

**Blood samples.** In blood samples the spectrum of expression again varied highly from other tissues, and was in several ways unexpected. Most of the chemokines displaying differential expression in other samples did not do so in blood. There was significant increase in expression of genes linked with antiviral response (OAS1, MX2, UBE1L, Isg15 and Bt.64826) on Days 1 and 8, and OAS1 remained upregulated through Day 22. The viperin-like potentially antiviral transcript (Bt.33922) was also strongly upregulated on Day 1 and continued through Day 15; however, none of the inducing interferons appeared increased in expression. Natural killer cell-associated genes displayed either no change or moderate downregulation. ICAM1 was moderately

upregulated, but other adhesion molecules were not. The antigen-presenting-cell-associated CD86 was increased in expression through Day 15, but neither of its corresponding ligands was differentially expressed. Neither complement components nor coagulation factors were differentially regulated. The neutrophil-associated calgranulin S100A12 was strongly downregulated at all time points. The gene Gprk5, which negatively regulates neutrophil chemotaxis[171,172], was strongly downregulated on Days 1 and 8. These data could suggest an increase of neutrophil transmigration into tissues. The macrophage marker CD68 and the macrophage lysosomal phospholipase A2[173] also had decreased expression in blood samples, possibly supporting a conclusion of macrophage migration into tissues.

There was remarkably little change in expression for most cytokines, with a few exceptions. In contrast to all other tissue types evaluated, IL1A was moderately downregulated in early infection. IL15 was downregulated at all time points. Interleukin 18 was strongly downregulated on Day 1 only. Neither of the characteristic T helper cytokine subsets was differentially expressed. IL15 and IL18 are both affiliated with the $T_H1$ response, as IL15 promotes development of $CD8^+$ memory cells and IL18 is known to induce IFNγ. The T cell receptor CD3 chains ε, γ and ζ all demonstrated decreases in transcription. The B cell ligand CD80 was also moderately decreased in expression. Immunoglobulin joining chain expression was increased through Day 15, but increases in immunoglobulin heavy or light chain transcripts were not detected. The B cell-associated gene TNFRSF5 (CD40), which is involved with immunoglobulin class switching, memory cell development and formation of germinal centers, was

upregulated at all time points. The gene TGFB1 was strongly increased at all time points. TGFβ is known to inhibit both B and T cell proliferation, and is involved in the antibody class switch to IgA. It is also implicated in the formation of regulatory or suppressor T cells[174,175]. The overall lack of acquired immune system activation detected in blood samples might argue toward the presence of regulatory T cells, but their characteristic mRNA transcripts (CD25, Foxp3) are similarly absent. Regulatory T cell involvement in mycoplasma infection has not been studied except with regard to the *M. arthriditis* superantigen, which has been found to exert an immunosuppressive effect through induction of a $CD4^+/CD8^-$ T cell population[176]. The spectrum of gene expression present in blood, while not unequivocal, fits a pattern of primarily B cell activation and immunoglobulin production with indications of immune suppression. This is partially in agreement with reports demonstrating a potent antibody response to infection with virulent MmmSC[69,89,177,178].

The pattern of gene expression seen in the blood samples was unanticipated. In a disease with a known septicemic component[179,180] and in animals clearly demonstrating systemic clinical signs it might be expected to observe strong upregulation of most genes associated with the immune system. This was clearly not the case, and a mechanism is not immediately apparent. In retrospect it may have been beneficial to extract leukocytes from blood prior to isolation of RNA. Although mature erythrocytes are anuclear, they maintain mRNA transcripts which may be isolated following phlebotomy[181]. As erythrocytes significantly outnumber leukocytes but have a limited repertoire of transcripts, assay of mRNA from whole blood may have biased the data

collection to reflect predominantly erythrocyte transcripts. Differential blood counts would also have contributed leukocyte population numbers, which might assist in interpreting the data.

In summary, temporal evaluation of MmmSC-induced gene expression in four different cattle tissues revealed activation of many genes involved with innate immunity but was less revealing for genes of the acquired immune system. A pattern of expression emerged indicating initiation of reaction to infection starting locally within the lower respiratory tract and later spreading to distant tissues. Bronchoalveolar lavage and bronchial biopsy tissues demonstrated an activation of cells of the monocyte/ macrophage lineage within the first day post-infection, along with numerous innate immune system genes usually associated with antiviral response. Signals expressed by BAL cells within the first day post-infection suggested recruitment of macrophages, neutrophils and natural killer cells out of the blood and into lung parenchyma, where their signals are obscured from our analysis after Day 8. Increased expression of adhesins local to the lower respiratory tract but not in peripheral blood facilitates neutrophil and monocyte transmigration into the lung. Solid evidence of $T_H1$ versus $T_H2$ activation was lacking in the tissues sampled as was concrete evidence of B cell activation, but indirect evidence suggested an early tendency toward $T_H1$ differentiation in the lower respiratory tract through Day 15 followed by a B cell response by Day 22. Complement and coagulation cascades were locally activated, and complement progressed from the mannose-binding pathway on Day 1 in BAL alone through classical pathway activation in both BAL and biopsy by Day 8 and evidence of membrane attack

complex formation in biopsy tissue by Day 15, indicating both a progression into deeper tissues and increased bactericidal activity coincident with disease resolution in surviving cattle. Lack of complement activation in nasal cytology samples upholds the lower respiratory predilection of MmmSC and further raises the possibility that lower respiratory tract complement activity may contribute toward the severe tissue damage seen in acute infection. Additional evidence of host contribution toward disease-induced tissue damage in the first week of infection is provided by increased expression of lysosomal transcripts in the lower respiratory tract, coincident with neutrophil infiltration. Proapoptotic transcripts were downregulated, and multiple transcription factors exhibited high expression.

KEGG pathway activation further demonstrated the wide-ranging response to MmmSC infection, particularly in the lower respiratory tract. The top-ranked BAL pathways were almost all associated with some aspect of the immune response. Bronchial biopsy pathways were similar to the BAL pathways and additionally displayed activation of pathways known to affect tissue damage such as oxidative phosphorylation, NK cell cytotoxicity and prostaglandin and leukotriene metabolism. This interweaving of the functions of BAL and biopsy pathways demonstrates the profound effects MmmSC infection has on the lower respiratory tract and provides additional candidate genes for host-induced self-tissue damage during MmmSC infection. Pathways activated in nasal cytology samples, while still part of the immune response, were not as proinflammatory as lower respiratory tract pathways. These pathways, present in a tissue with little inflammation, suggest that other pathways should be explored first

when seeking host genes exploited by MmmSC to cause tissue damage. Blood pathways remain cryptic; while some are clearly affiliated with endothelial cell metabolism, the association of others with the response to infection is undetermined. While many of the top-ranked pathways in each tissue were affiliated with the immune or inflammatory response, a number of pathways apparently unrelated to immune response also showed strong differential expression. These included several involving hormone and amino acid metabolism, lipid metabolism, insulin and diabetes, and ethylbenzene degradation; further work will be needed to determine their contribution to disease pathogenesis.

Most differentially-expressed host genes during MmmSC infection had strong alterations in expression on the first sampling day post-infection followed by a gradual return toward baseline expression. Additionally, the spectrum of gene activation was quite different in each tissue although functions often complemented each other, especially between BAL and biopsy samples. Future studies might augment this information with targeted collection of lymphoid tissues and/or simultaneous evaluation of secreted gene products to better determine the involvement of lymphocytes. Application of other techniques such as laser capture microdissection or proteome analysis may also assist in better elucidating the nature of the host response to infection.

# CHAPTER IV

## COMPARATIVE STRAIN AND SURVIVOR ANALYSIS IN CATTLE INFECTED WITH *MYCOPLASMA MYCOIDES MYCOIDES* SMALL COLONY

### INTRODUCTION

*Mycoplasma mycoides mycoides* small colony (MmmSC) has been found to affect the expression of a multitude of genes in the bovine host, but the mechanism by which each gene influences the outcome of infection is unknown. Global gene expression was investigated using microarrays to detect mRNA transcripts, but this method is only semiquantitative. Microarray results must be confirmed with a more sensitive method such as real-time polymerase chain reaction (RT-PCR). The purpose of this segment of the experiment was to directly compare gene expression of selected genes across all tissues and time points using RT-PCR following infection with one of three strains of MmmSC. The second objective was to compare selected genes between Gladysdale-infected survivors and nonsurvivors to determine if any of the selected genes appeared to influence host survival.

### MATERIALS AND METHODS

For this portion of the experiment, the animals, inoculum, and clinical samples collected were the same as those presented in Chapter II, using MmmSC strains

Gladysdale, Ondangwa and Shawawa. RNA was extracted from clinical samples using the protocol described in Chapter III.

**Reverse transcription of RNA.** For the strain comparison, pooled samples were used. Each individual animal contributed 0.4 μg of purified RNA to create a pooled aliquot of 2 μg RNA. The aliquots were reverse transcribed into cDNA using TaqMan (Applied Biosystems). For relative quantitation of target cDNA, samples were run in duplicate in individual tubes in a SmartCycler I (Cepheid). One SmartMix bead (Cepheid) was used for 2 x 25 μl PCR reactions along with 20 ng of cDNA, 0.2X SYBR Green I dye (Invitrogen) and 0.3 μM forward and reverse primers (Sigma Genosys) designed by Primer Express Software v2.0 (Applied Biosystems) or Primer3Plus (www.bioinformatics.nl/cgi-bin/primer3plus/primer3plus.cgi) (**Table 8**). Not all genes in Table 8 were evaluated in each comparison. For each gene tested, the individual calculated threshold cycles (Ct) were averaged among each condition and normalized to the Ct of the bovine β2-microglobulin gene from the same cDNA samples before calculating the fold change using the $\Delta\Delta C_t$ method (Applied Biosystems Prism SDS 7700 User Bulletin #2). Due to the nature of this calculation in which all results were averaged prior to determination of $\Delta\Delta C_t$, no error bars could be generated. For each primer pair, a negative control (water) and an RNA sample without reverse transcriptase (to determine genomic DNA contamination) were included as controls during cDNA quantitation.

For the survivor-nonsurvivor comparison, only strain Gladysdale was used. Animals were divided into two groups: nonsurvivors included the two animals

euthanized early in the experiment, while survivors included the other three animals that resolved infection and were euthanized on Day 36. Individual animal cDNA was transcribed using TaqMan as described above, then group aliquots were created by combining equal amounts of individual animal cDNA. Comparisons were conducted only for Days 1 and 8, as nonsurvivors were euthanized prior to Day 15.

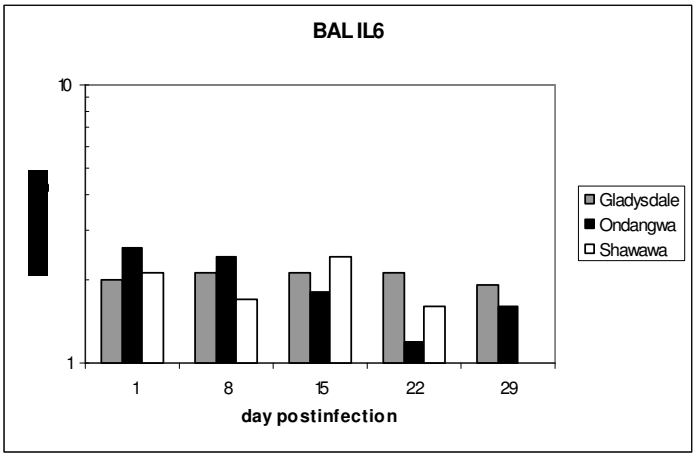
## RESULTS

Nine genes were compared between strains Gladysdale, Ondangwa and Shawawa. Gene expression was compared in bronchoalveolar lavage fluid, bronchial biopsy, and nasal cytology brushings. Blood was not compared as insufficient RNA was obtained from Ondangwa and Shawawa-infected animals' blood samples. The genes compared included interleukin 6 (IL6), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA), interleukin 18 (IL18), calmodulin 2 (CALM2), ferritin heavy polypeptide 1 (FTH1), interleukin 8 (IL8), mitogen activated protein kinase 1 (MAPK1), interleukin 1B (IL1B), and signal transducer and activator of transcription 3 (STAT3). Genes were selected based on early Bayesian modeling of microarray data suggesting that each gene might be involved in disease pathogenesis in at least one tissue type.

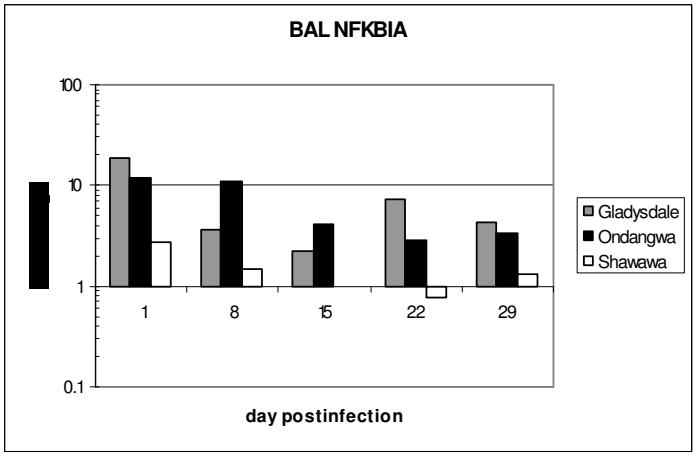
**Bronchoalveolar lavage strain comparison.** Real-time PCR results of BAL samples showed no major differences between the three strains for genes IL6 or CALM2 (**Figure 28**). The Shawawa strain demonstrated much less induction of NFKBIA in

**Figure 28. RT-PCR fold changes for bronchoalveolar lavage.** A. gene interleukin 6 (IL6); B. gene nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA); C. gene interleukin 18 (IL18); D. gene calmodulin 2 (CALM2); E. gene ferritin heavy polypeptide 1 (FTH1); F. gene interleukin 8 (IL8); G. gene mitogen activated protein kinase 1 (MAPK1); H. gene interleukin 1B (IL1B); I. gene signal transducer and activator of transcription 3 (STAT3).

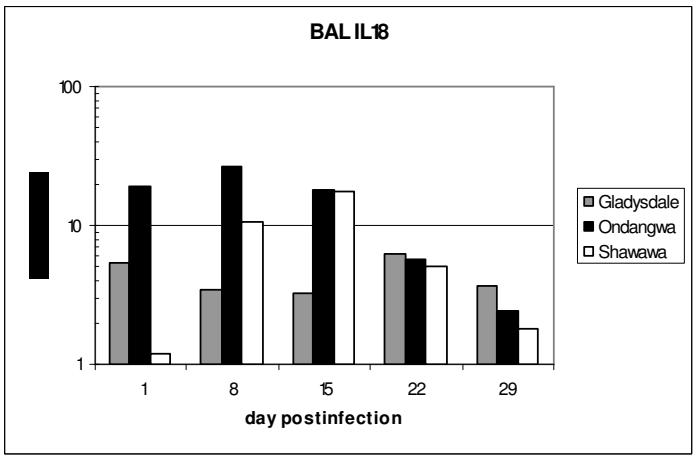
**A**


BAL IL6
10
1
1
8
15
22
29
day postinfection
Gladysdale
Ondangwa
Shawawa

**B**


BAL NFKBIA
100
10
1
0.1
1
8
15
22
29
day postinfection
Gladysdale
Ondangwa
Shawawa

**C**


BAL IL18
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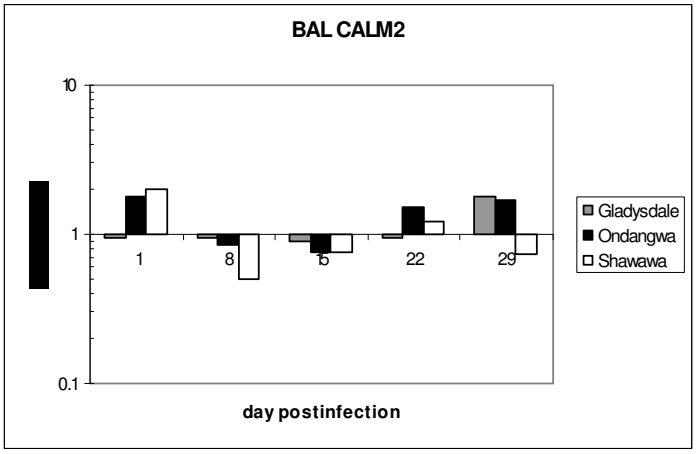
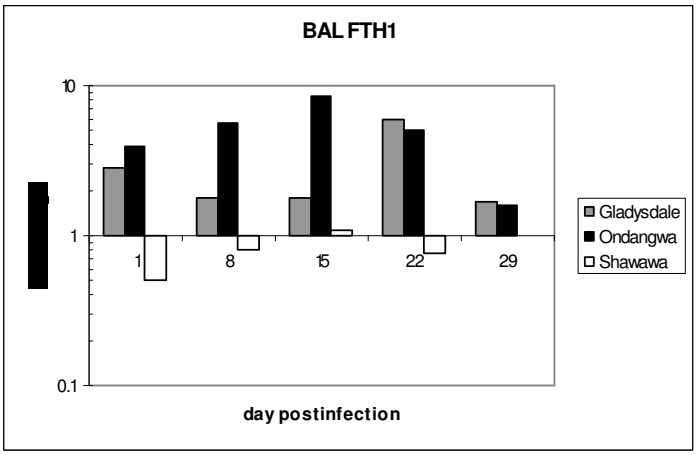
10
1
1
8
15
22
29
day postinfection
Gladysdale
Ondangwa
Shawawa

**D**




**E**




**F**

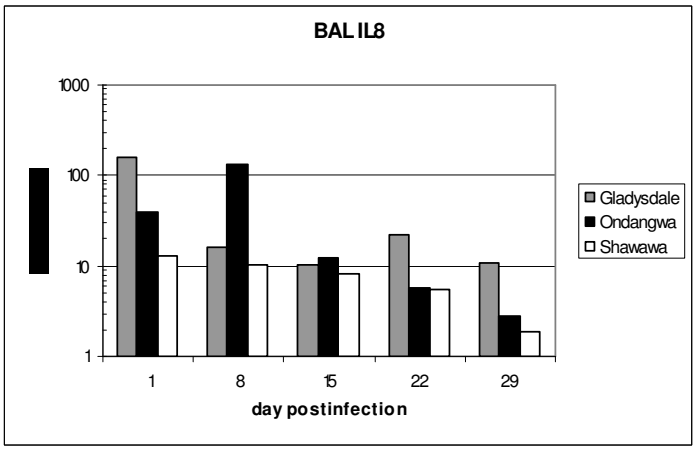
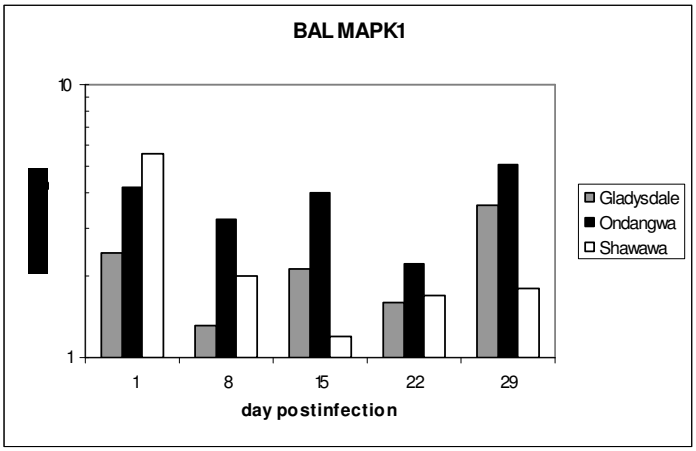
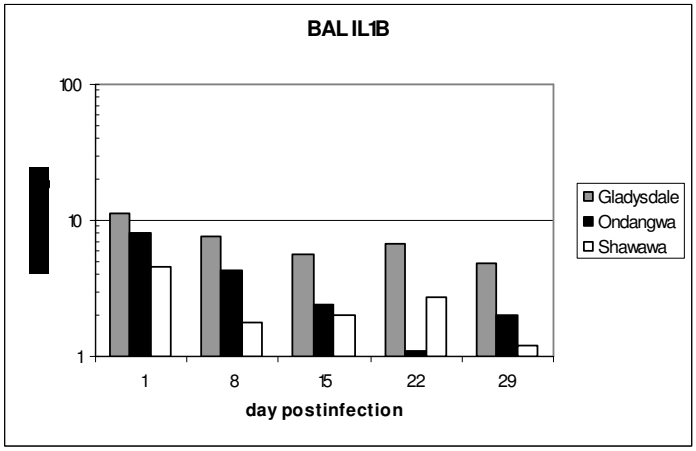



**Figure 28 continued**

**G**




**H**




**I**

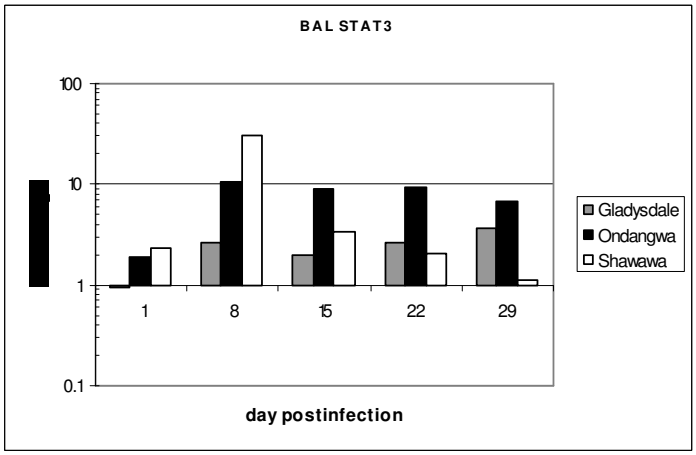


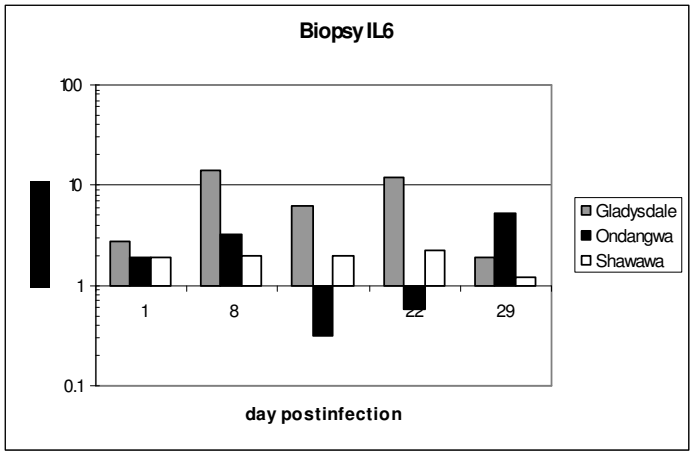

**Figure 28 continued**

infected cattle than Gladysdale or Ondangwa. Infection with either Ondangwa or Shawawa induced greater transcription of IL18 than infection with Gladysdale, particularly from Days 1-15. For FTH1, Ondangwa-infected cattle showed the highest levels of transcription, with Shawawa-infected animals exhibiting very low levels. Interleukin 8 expression was dramatically increased for both Gladysdale and Ondangwa-infected animals, but peak expression differed temporally between the strains. Shawawa also demonstrated increased IL8 expression, but not to the extent of the other strains. Expression of MAPK1 was highest in animals infected with strain Ondangwa, but the differences were not large. Gladysdale-infected animals showed the greatest expression of IL1B, but all strains induced increases in expression. Both Ondangwa and Shawawa-infected animals demonstrated greater transcription of STAT3 than Gladysdale-infected animals starting on Day 8.

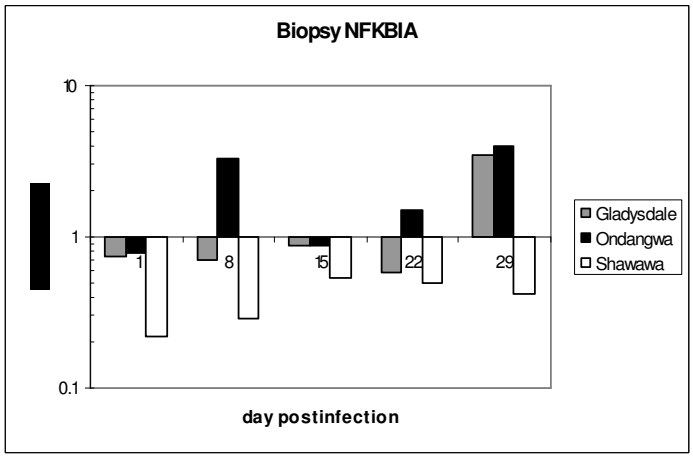
**Bronchial biopsy strain comparison.** Interleukin 6 induction was greater in Gladysdale-infected animals (**Figure 29**). All animals showed decreases in NFKBIA expression, but Shawawa-infected cattle demonstrated dramatic downregulation in early infection. Interleukin 18 expression was increased in Ondangwa-infected animals but decreased below baseline levels in animals infected with the other strains. All infected animals showed downregulation of CALM2, with Ondangwa-infected cattle showing the greatest decrease in expression. All infected animals showed increases in IL8 expression on Day 1, but only Ondangwa-infected animals continued to demonstrate increased expression through Day 8. MAPK1 expression was increased in all groups, but

**Figure 29. RT-PCR fold changes for bronchial epithelial cells.** A. gene interleukin 6 (IL6); B. gene nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA); C. gene interleukin 18 (IL18); D. gene calmodulin 2 (CALM2); E. gene ferritin heavy polypeptide 1 (FTH1); F. gene interleukin 8 (IL8); G. gene mitogen activated protein kinase 1 (MAPK1); H. gene interleukin 1B (IL1B); I. gene signal transducer and activator of transcription 3 (STAT3).

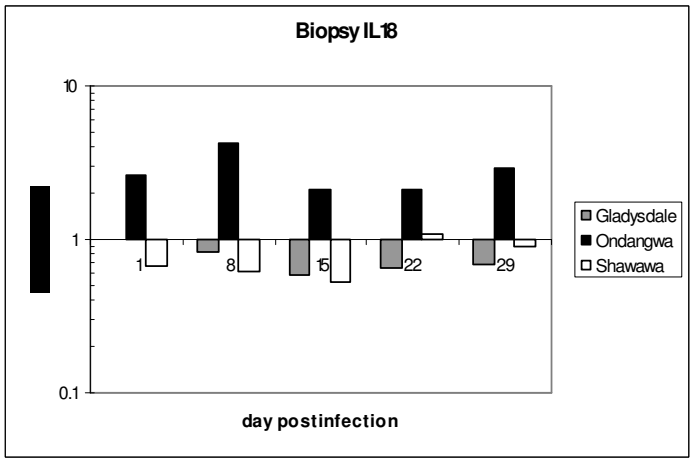
**A**


Biopsy IL6
100
10
1
0.1
1
8
22
29
day postinfection
Gladysdale
Ondangwa
Shawawa

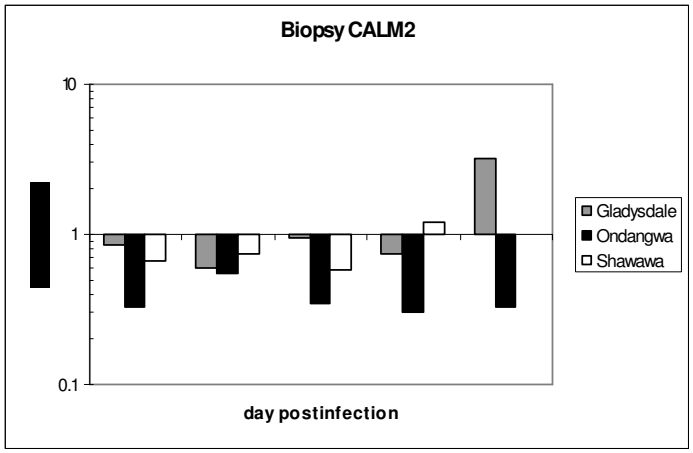
**B**


Biopsy NFKBIA
10
1
0.1
1
8
15
22
29
day postinfection
Gladysdale
Ondangwa
Shawawa

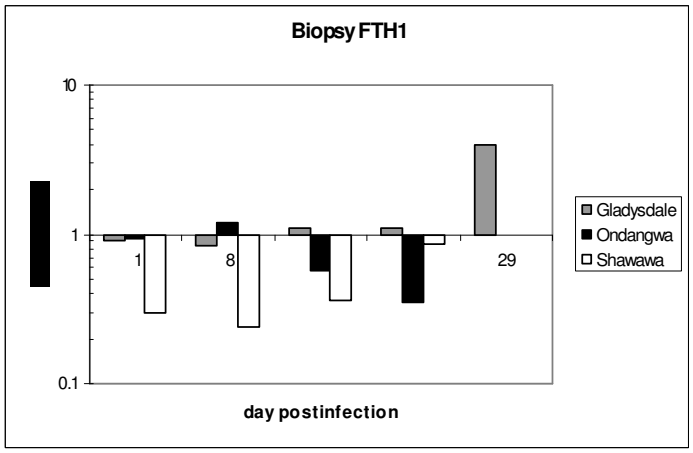
**C**


Biopsy IL18
10
1
0.1
1
8
15
22
29
day postinfection
Gladysdale
Ondangwa
Shawawa

**D**




**E**




**F**

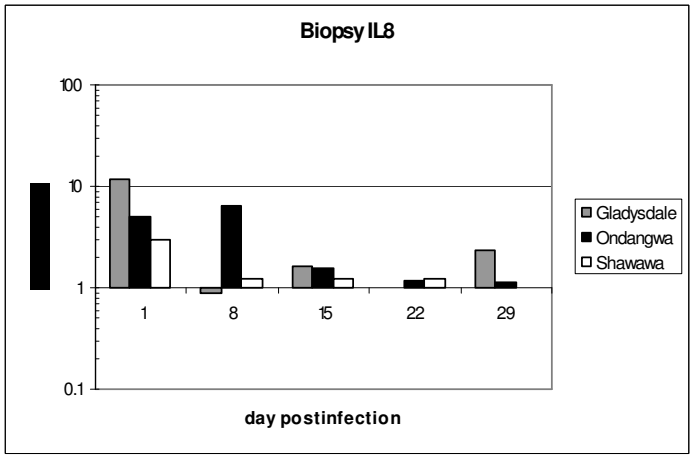
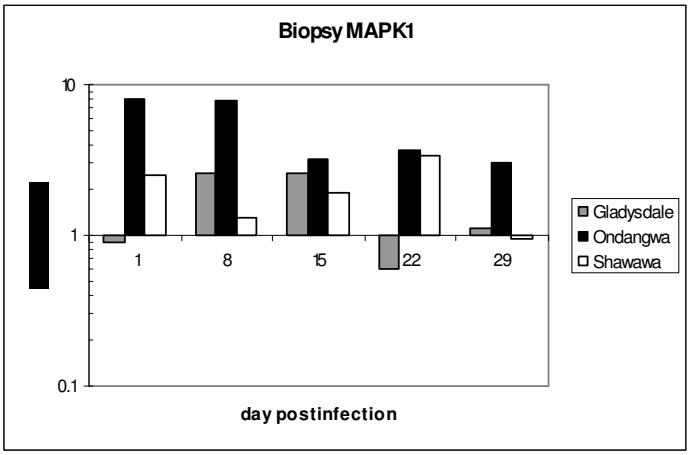
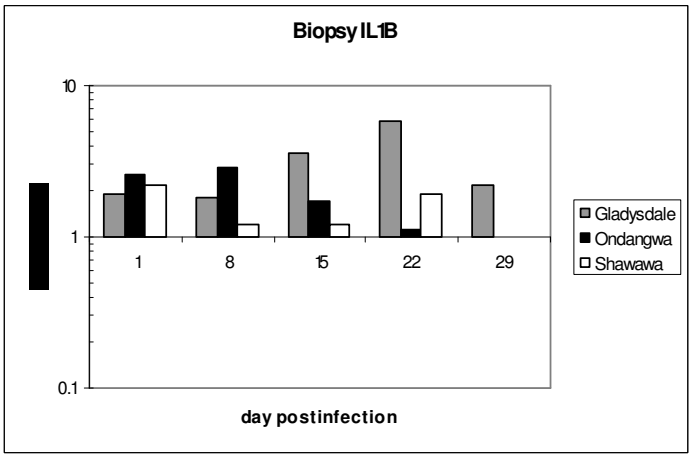



**Figure 29 continued**

**G**




**H**




**I**

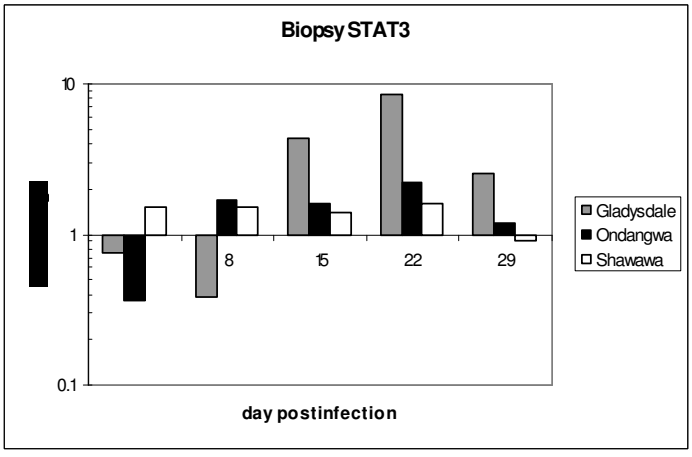


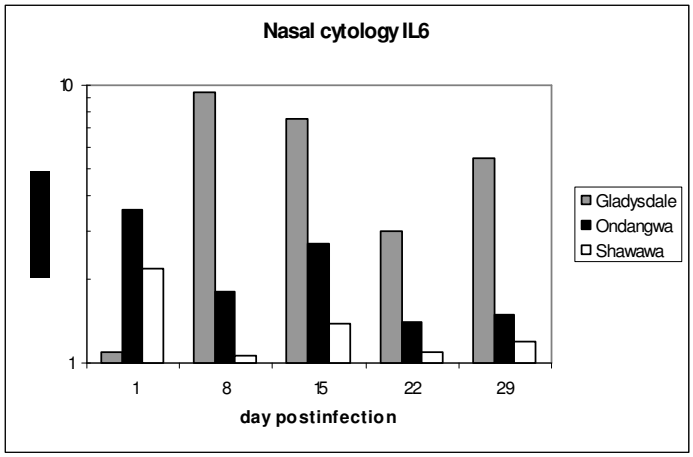

**Figure 29 continued**

Ondangwa-infected cattle showed higher expression from Days 1-8. For IL1B, expression in Ondangwa- and Shawawa-infected animals was only slightly increased, but Gladysdale-infected animals showed a sustained increase peaking on Day 22. STAT3 expression was downregulated from Days 1-8 but upregulated from Days 15-29 in Gladysdale-infected animals. Ondangwa-infected animals showed downregulation only on Day 1, and Shawawa-infected animals showed virtually no change from baseline.

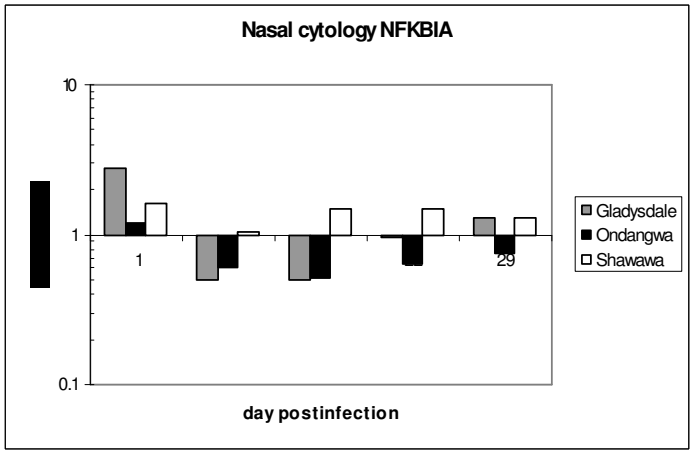
**Nasal cytology strain comparison.** Interleukin 6 expression in Gladysdale-infected animals was greatly increased over the other strains (**Figure 30**). Ondangwa-infected animals showed moderate increases over baseline, while Shawawa-infected animals showed almost no change. No differences were noted in NFKBIA expression between strains. Only Shawawa-infected animals demonstrated changes in expression in IL18; a moderate increase was noted early in infection. Shawawa-infected cattle also showed a Day 1 increase in CALM2 expression while animals infected with other strains did not. FTH1 expression did not differ between strains and was never far from baseline. All strains induced increases in IL8 expression in the host animal on Day 1, but only Shawawa-infected animals continued the increased expression through Day 8. MAPK1 expression did not vary greatly from baseline, and no difference was seen between strains. All strains induced an early increase in IL1B expression, but only Gladysdale induced IL1B downregulation later in infection. No strain differences were noted in STAT3 expression, which never altered greatly from baseline.

**Figure 30. RT-PCR fold changes for nasal epithelial cells.** A. gene interleukin 6 (IL6); B. gene nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA); C. gene interleukin 18 (IL18); D. gene calmodulin 2 (CALM2); E. gene ferritin heavy polypeptide 1 (FTH1); F. gene interleukin 8 (IL8); G. gene mitogen activated protein kinase 1 (MAPK1); H. gene interleukin 1B (IL1B); I. gene signal transducer and activator of transcription 3 (STAT3).

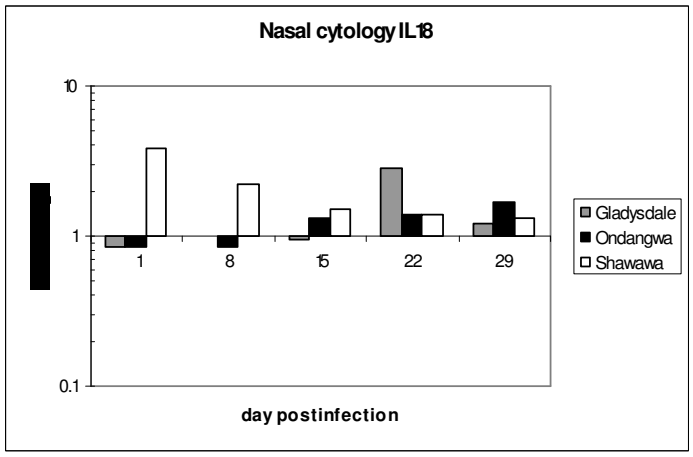
**A**


Nasal cytology IL6
10
1
1
8
15
22
29
day postinfection
Gladysdale
Ondangwa
Shawawa

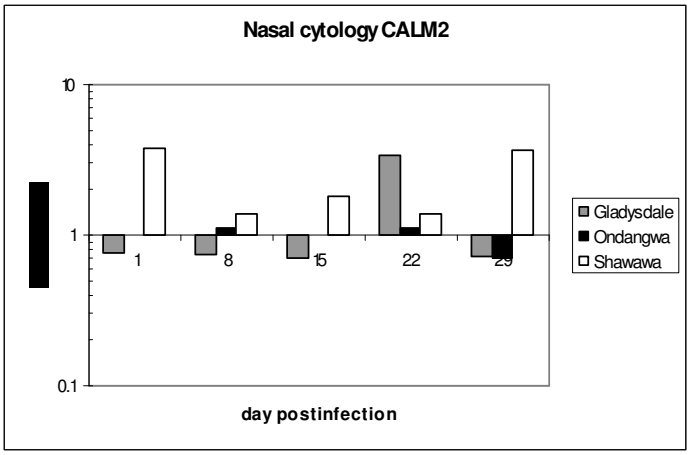
**B**


Nasal cytology NFKBIA
10
1
0.1
1
29
day postinfection
Gladysdale
Ondangwa
Shawawa

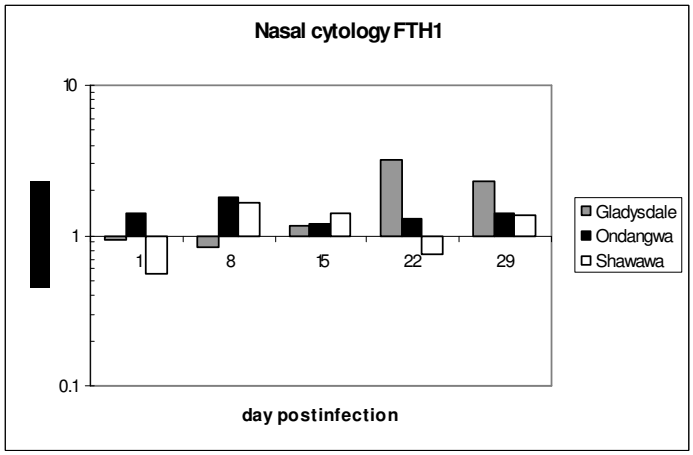
**C**


Nasal cytology IL18
10
1
0.1
1
8
15
22
29
day postinfection
Gladysdale
Ondangwa
Shawawa

**D**




**E**




**F**

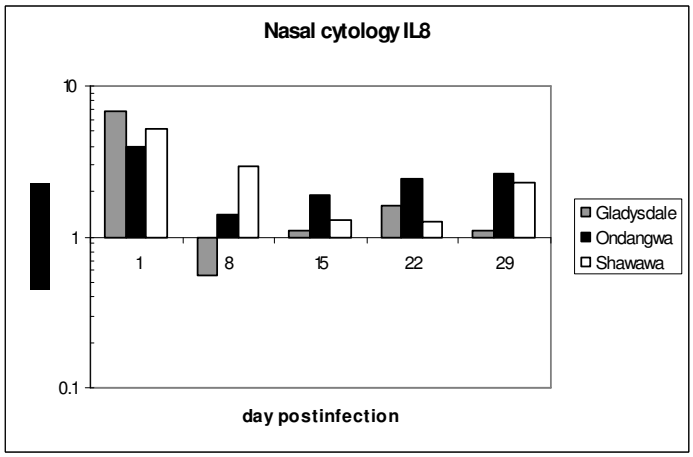
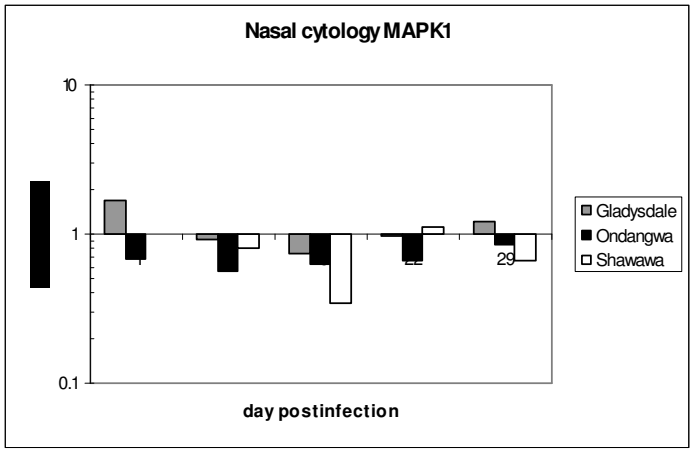
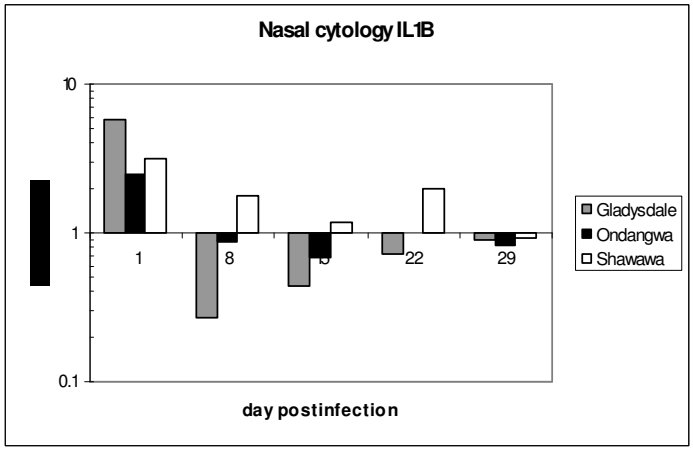



**Figure 30 continued**

**G**




**H**




**I**

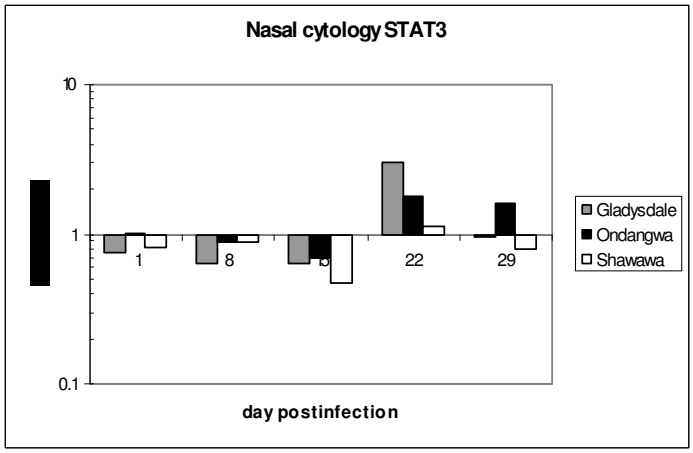



**Figure 30 continued**

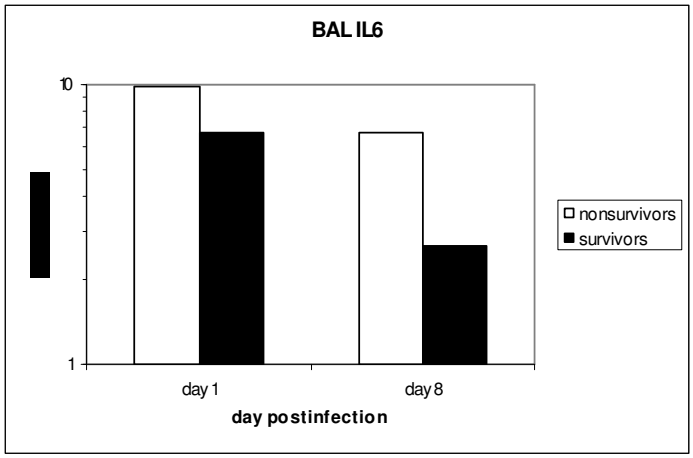
**Survivor-nonsurvivor comparisons.** Gene expression in the three Gladysdale-infected animals that survived until Day 36 was compared to gene expression in the two animals that were euthanized on Days 10 and 13 due to severe disease. Six genes were compared: interleukin 6 (IL6), nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA), interleukin 18 (IL18), calmodulin 2 (CALM2), ferritin heavy polypeptide 1 (FTH1), and interleukin 8 (IL8).

**Bronchoalveolar lavage survivor comparison.** Nonsurviving animals showed IL6 expression that was one-third greater than surviving animals on Day 1, but was threefold that of surviving animals on Day 8 (**Figure 31**). Similarly, expression of NFKBIA in bronchoalveolar lavage of nonsurvivors was threefold that of survivors on Day 1 and twice that of survivors on Day 8. Expression of interleukin 18 on Day 1in nonsurvivors was less than half that of survivors, but this difference had diminished by Day 8. Expression of CALM2 was not greatly different between groups on Day 1, but on Day 8 nonsurvivors demonstrated a fivefold decrease in expression when compared to survivors. No difference between groups was seen in expression of FTH1. While all animals demonstrated profound induction of IL8 expression, less than twofold difference was seen between surviving and nonsurviving cattle.

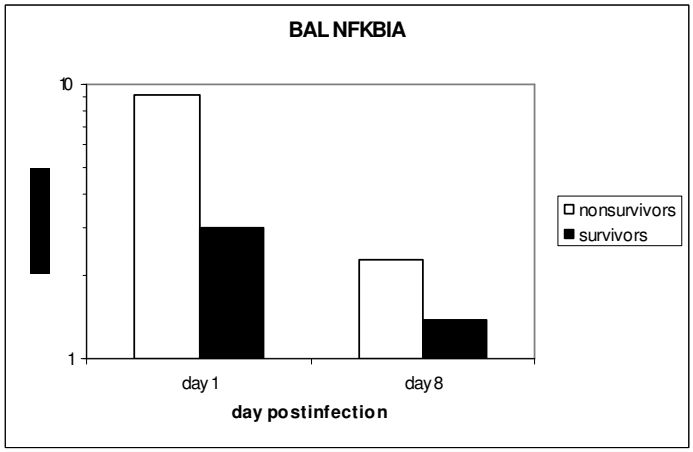
**Bronchial biopsy survivor comparison.** Both groups showed large increases in IL6 gene expression on Day 8, but there was no difference between the groups (**Figure 32**). Similarly, there was no discernable difference in NFKBIA expression, IL18 expression, CALM2 expression or FTH1 expression. Interleukin 8 evaluation did show

**Figure 31. Survivor vs. nonsurvivor gene expression in bronchoalveolar lavage.** A. gene interleukin 6 (IL6); B. gene nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA); C. gene interleukin 18 (IL18); D. gene calmodulin 2 (CALM2); E. gene ferritin heavy polypeptide 1 (FTH1); F. gene interleukin 8 (IL8).

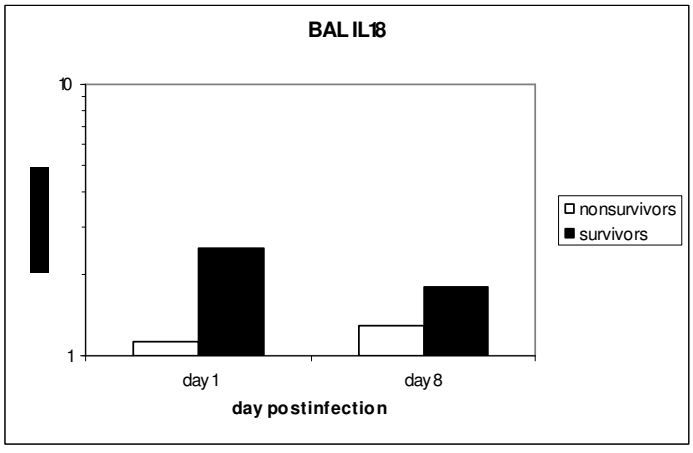
**A**


BAL IL6
10
1
day 1
day 8
day postinfection
nonsurvivors
survivors

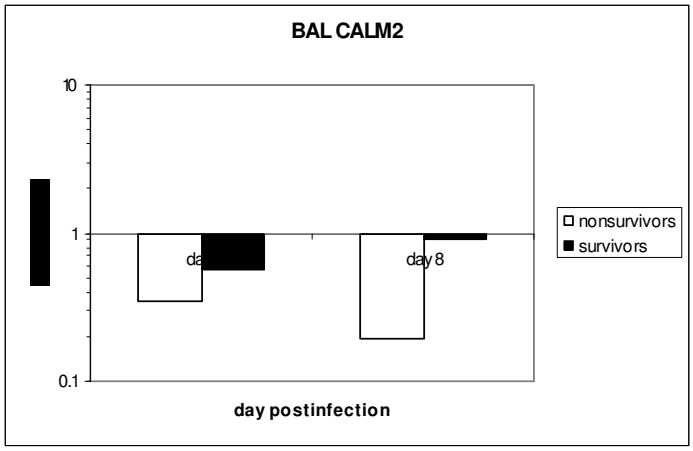
**B**


BAL NFKBIA
10
1
day 1
day 8
day postinfection
nonsurvivors
survivors

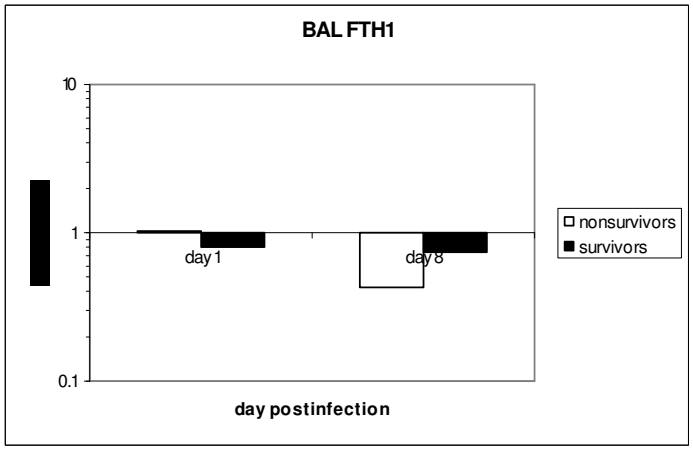
**C**


BAL IL18
10
1
day 1
day 8
day postinfection
nonsurvivors
survivors

**D**




**E**




**F**

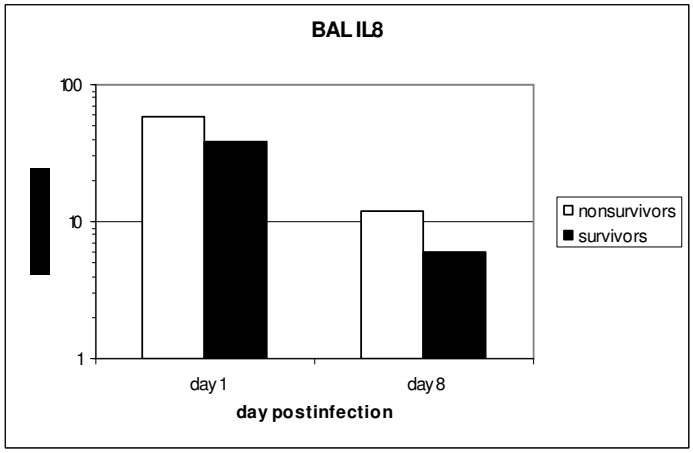
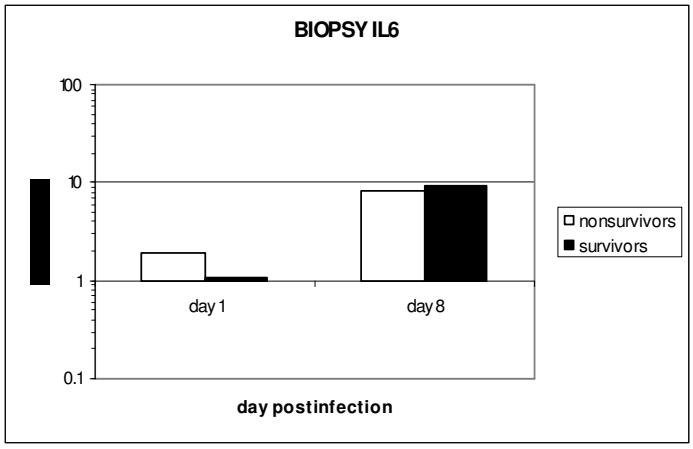


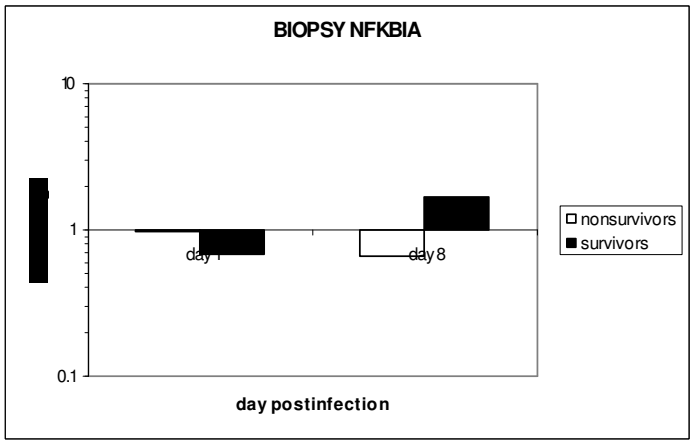

**Figure 31 continued**

**Figure 32. Survivor vs. nonsurvivor gene expression in bronchial epithelial cells.** A. gene interleukin 6 (IL6); B. gene nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA); C. gene interleukin 18 (IL18); D. gene calmodulin 2 (CALM2); E. gene ferritin heavy polypeptide 1 (FTH1); F. gene interleukin 8 (IL8).

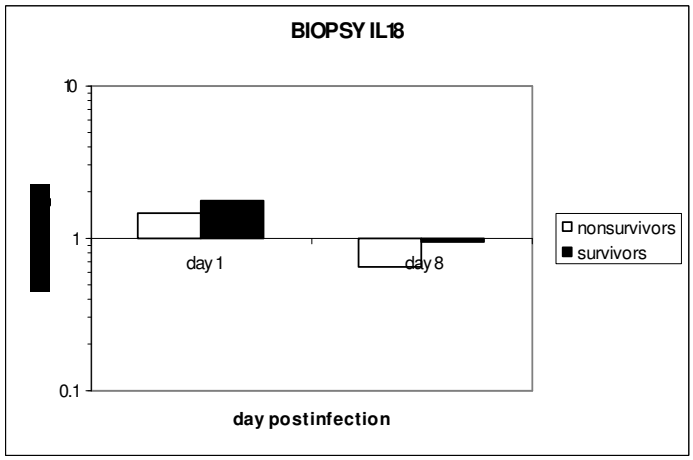
**A**


BIOPSY IL6
100
10
1
0.1
day 1
day 8
nonsurvivors
survivors
day postinfection

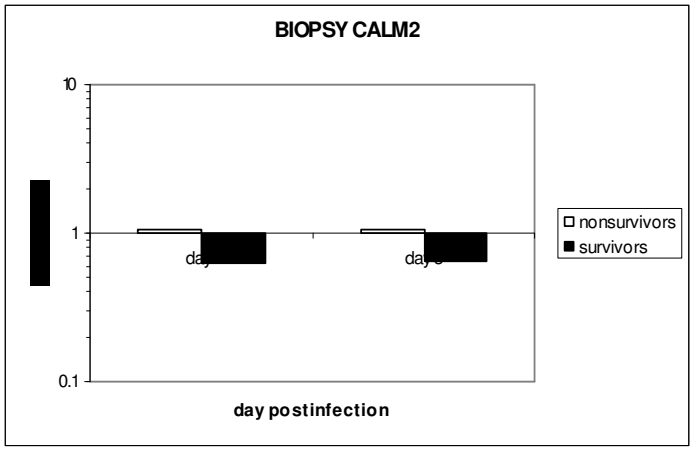
**B**


BIOPSY NFKBIA
10
1
0.1
day 1
day 8
nonsurvivors
survivors
day postinfection

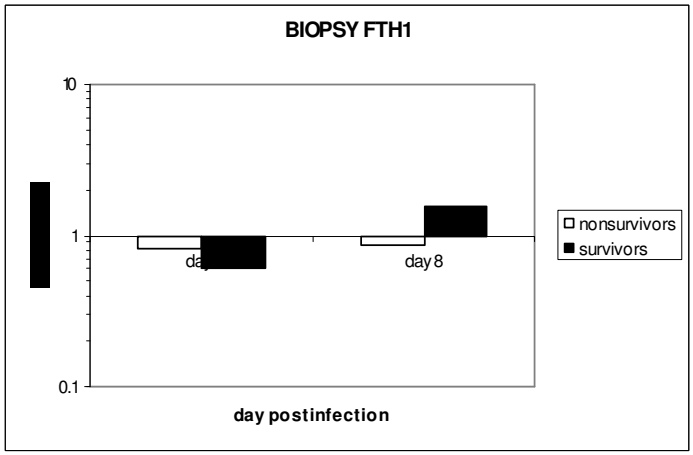
**C**


BIOPSY IL18
10
1
0.1
day 1
day 8
nonsurvivors
survivors
day postinfection

**D**




**E**




**F**

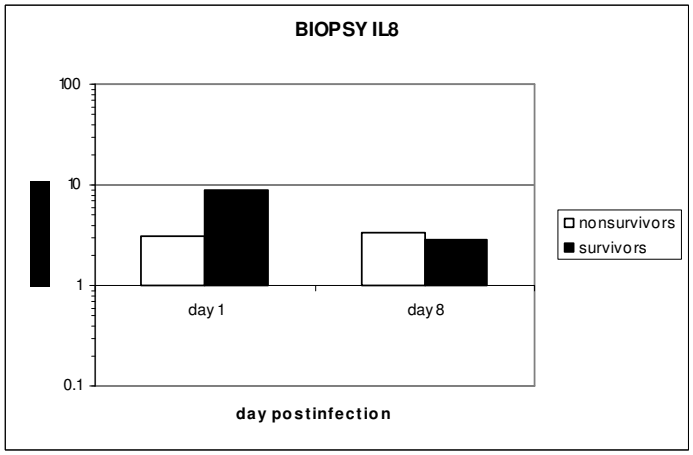
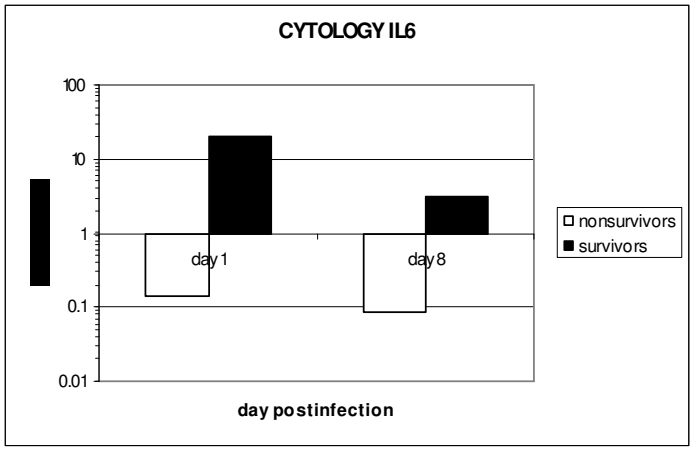


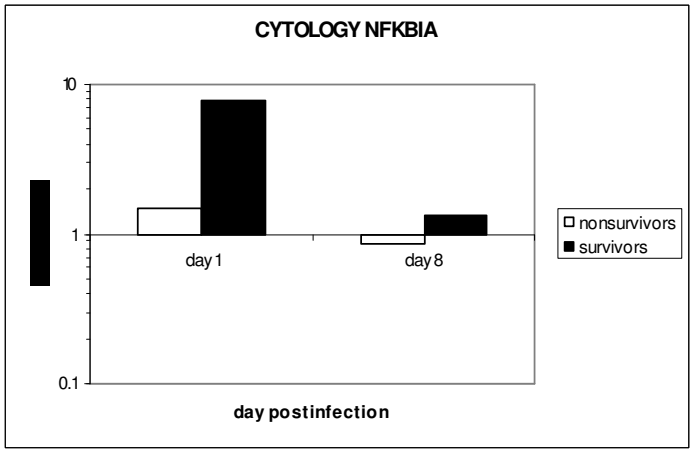

**Figure 32 continued**

**Figure 33. Survivor vs. nonsurvivor gene expression in nasal epithelial cells.** A. gene interleukin 6 (IL6); B. gene nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA); C. gene interleukin 18 (IL18); D. gene calmodulin 2 (CALM2); E. gene ferritin heavy polypeptide 1 (FTH1); F. gene interleukin 8 (IL8).

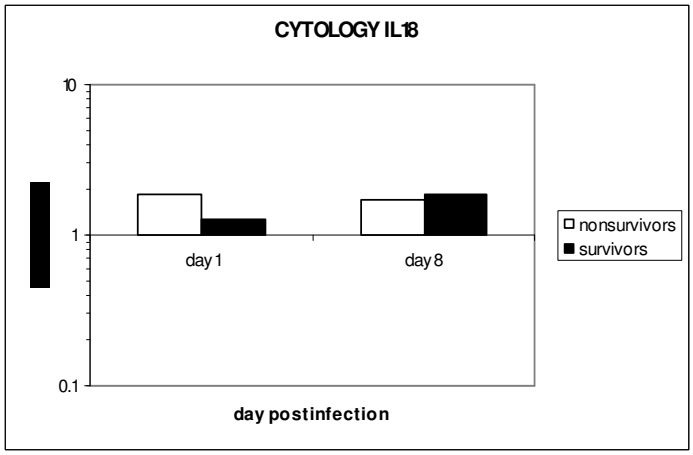
**A**


CYTOLOGY IL6
100
10
1
0.1
0.01
day 1
day 8
nonsurvivors
survivors
day postinfection

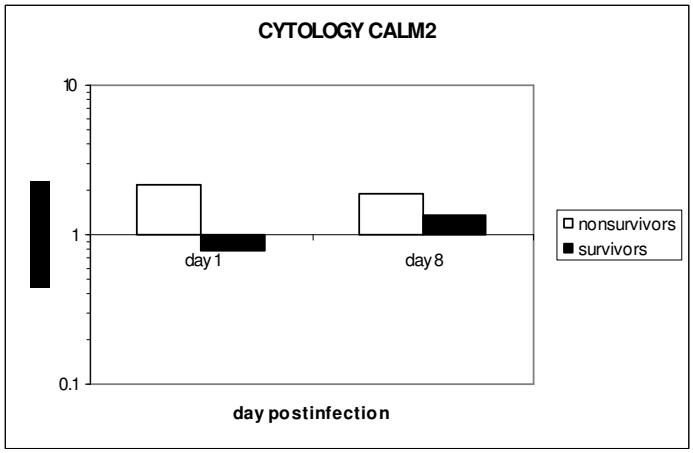
**B**


CYTOLOGY NFKBIA
10
1
0.1
day 1
day 8
nonsurvivors
survivors
day postinfection

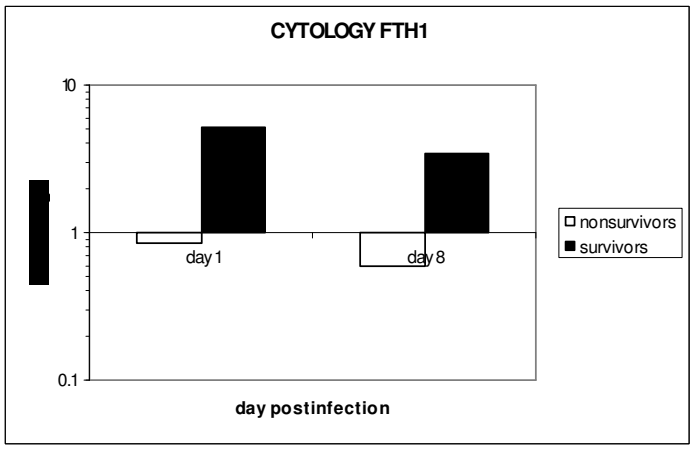
**C**


CYTOLOGY IL18
10
1
0.1
day 1
day 8
nonsurvivors
survivors
day postinfection

**D**




**E**




**F**

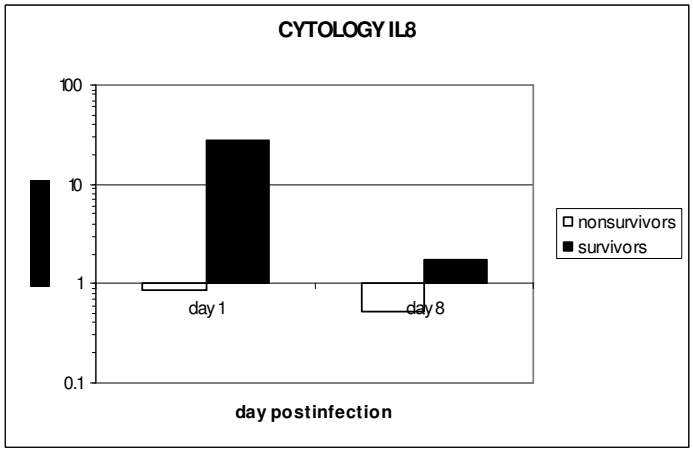
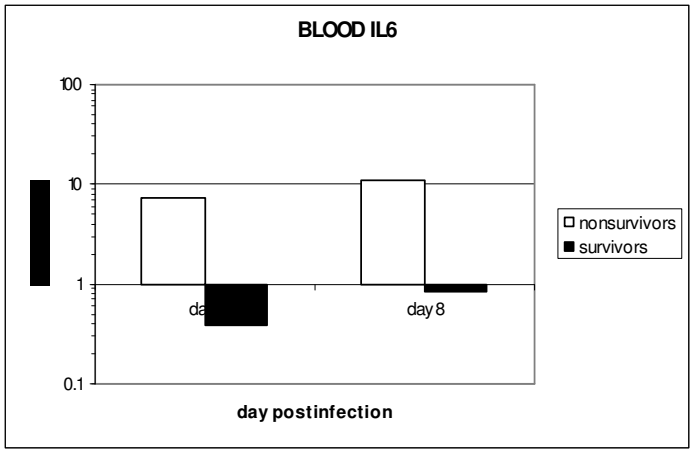


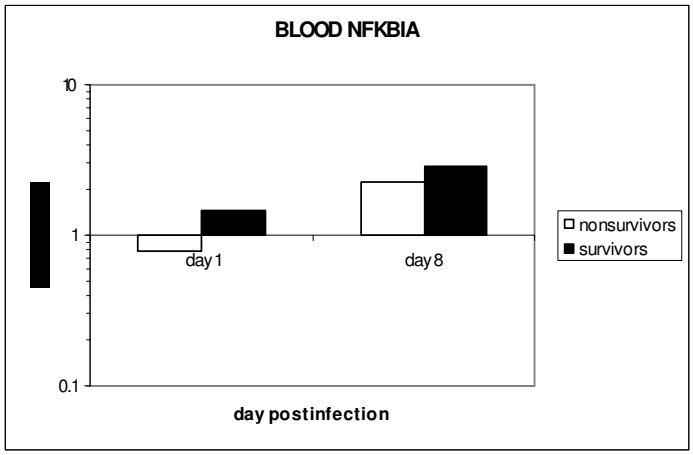

**Figure 33 continued**

**Figure 34. Survivor vs. nonsurvivor gene expression in blood.** A. gene interleukin 6 (IL6); B. gene nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA); C. gene interleukin 18 (IL18); D. gene calmodulin 2 (CALM2); E. gene ferritin heavy polypeptide 1 (FTH1); F. gene interleukin 8 (IL8).

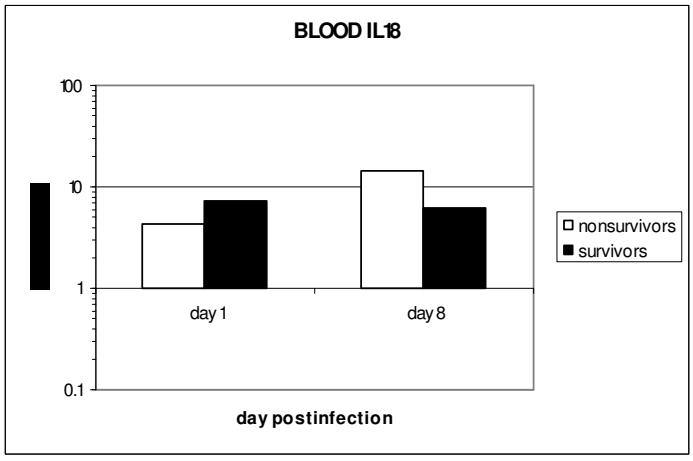
**A**


BLOOD IL6
100
10
1
0.1
day 8
nonsurvivors
survivors
day postinfection

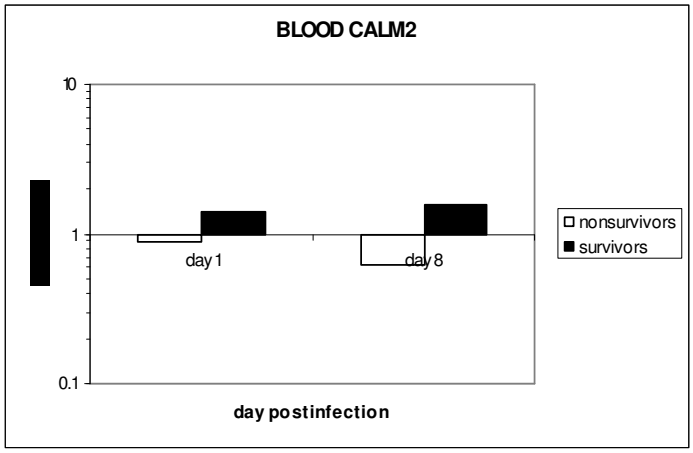
**B**


BLOOD NFKBIA
10
1
0.1
day 1
day 8
nonsurvivors
survivors
day postinfection

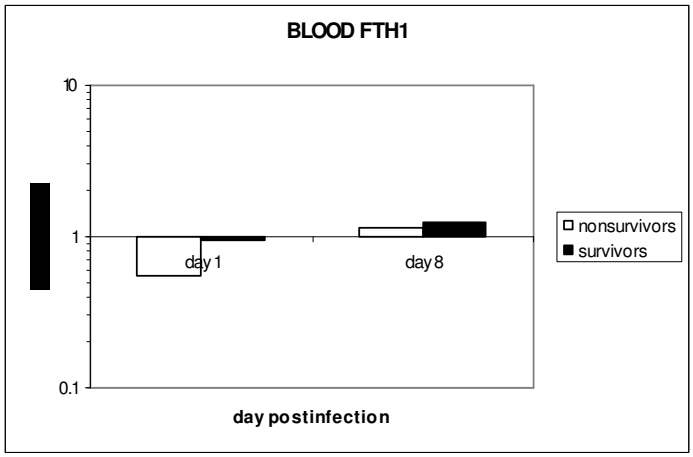
**C**


BLOOD IL18
100
10
1
0.1
day 1
day 8
nonsurvivors
survivors
day postinfection

**D**




**E**




**F**

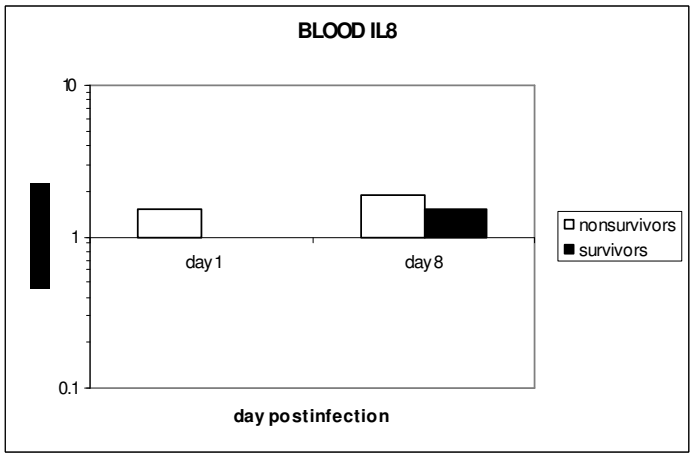



**Figure 34 continued**

a threefold greater expression in surviving animals over nonsurvivors on Day 1, but this difference had vanished by Day 8.

**Nasal cytology survivor comparison.** Evaluation of IL6 expression demonstrated a profound difference between survivors and nonsurvivors in which survivors showed a more than twentyfold increase in expression on Day 1 and more than tenfold on Day 8 (**Figure 33**). Similarly, a fivefold greater NFKBIA expression was seen in survivors on Day 1, although that difference had disappeared by Day 8. No large differences were noted in expression of IL18 or CALM2 between groups. For FTH1, survivors demonstrated a fourfold increase in expression over nonsurvivors on both Day 1 and Day 8. Expression of IL8 was more than twentyfold greater in survivors on Day 1, but no difference remained on Day 8.

**Blood survivor comparison.** Interleukin 6 expression was more than sixfold greater in nonsurvivors on both Day 1 and Day 8 (**Figure 34**). No major differences were noted between groups for NFKBIA, IL18, CALM2, FTH1 or IL8 expression.

## DISCUSSION

Differences in disease severity, morbidity and mortality between strains of MmmSC are well-recognized. The most prominent factor influencing differential severity based on published literature would appear to be assignation of a given MmmSC strain into one of the two major clusters, African/Australian or European[182]. African/Australian isolates are reported to demonstrate significantly higher morbidity and mortality over European isolates[183]. There is an 8.8-kb deletion in the European

cluster, and several investigations have been conducted in that region in search of virulence factors differentiating the African/ Australian and European strains. Sequencing of the deletion area present in the African strains has been conducted and has identified five open reading frames including the membrane lipoprotein *LppB* and the glycerol transport cassette components *gtsB* and *gtsC*[43-46,184,185].

Studies comparing the host response to infection with different strains of MmmSC have previously focused on detection of secreted products or mitogen activation[53,64,65,69,97]. No studies have been published evaluating host gene expression for any organism in the mycoides cluster. The current study used real-time polymerase chain reaction to investigate the expression of selected genes in the bovine host after infection with one of three African/Australian strains of MmmSC. The clinical results seen in this study demonstrated Gladysdale as the most virulent isolate, followed by Ondangwa, and Shawawa as the least virulent of the three strains.

Interleukin 6 is a multifunctional pyrogenic cytokine secreted by activated macrophages. It is known to stimulate innate immunity by inducing synthesis of hepatic acute-phase proteins, increasing vascular permeability and coagulation and activating both T and B cells. Its induction in cases of human mycoplasmal pneumonia has been described[186], but an experiment involving *M. hyopneumoniae* failed to find increased soluble IL6 in culture supernatant[161]. A murine study linked pulmonary IL6 expression with mortality in polymicrobial sepsis[187] and suggested that IL6 and its transcription factors could play a role in the initiation and propagation of acute lung injury. The small increase in IL6 gene expression seen in BAL fluid with all three strains of MmmSC

suggests that this cytokine is not a significant mediator of differential disease severity, since the predominantly macrophage population of BAL fluid should be a significant source of IL6. However, the prominent increase in IL6 mRNA transcription over baseline levels in bronchial biopsy and nasal cytology samples from Gladysdale-infected animals argues for a role for this cytokine. IL6 in these samples would be expected to result from the inclusion of tissue macrophages within the bronchial or nasal epithelium. Since activation of macrophages is usually accomplished via phagocytosis of particulate antigen (the mycoplasma), it is difficult to comprehend a scenario in which embedded tissue macrophages would encounter the mycoplasma before the alveolar macrophages. One possible explanation might be that alveolar macrophages may already be in an activated state, so there is no apparent increase in cytokine secretion over baseline. Potential inciting stimuli for alveolar exposure to particulate antigen and activation of alveolar macrophages in this experiment include confinement housing and the endoscopic lavage procedure. Epithelial macrophages might be more protected from ready access to environmental antigens and could have a lower baseline expression of cytokines, making the differential expression upon exposure more prominent. Regardless of the method of macrophage activation, the differential expression of this cytokine by Gladysdale-infected cattle does suggest its involvement in disease severity.

Nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha (NFKBIA) is an inhibitor of the transcription factor complex NFKB. Activated NFKB complex translocates into the nucleus and binds DNA at kappa B binding motifs. NFKBIA, an I kappa B protein, inactivates NFKB by trapping it in the cell cytoplasm.

Phosphorylation of serine residues on the I kappa B proteins by kinases marks them for destruction via the ubiquitination pathway, allowing reactivation of the NFKB complex. NFKB is involved in the transcription of many cytokines, and has been implicated in the pathogenesis of diseases in several species. Mycoplasmal lipoproteins have been shown to activate NFKB via TLR signaling[188-190]. Increased expression of NFKBIA has been described in human sepsis[191], but not in any mycoplasmal diseases. Induction of NFKBIA and inhibition of the cytokine-transcribing NFKB might be expected to increase the severity of disease by hamstringing the host immune response. Alternatively, inhibition of host proinflammatory cytokines may ameliorate tissue damage caused by overwhelming immune system activation. Strain Gladysdale, the most pathogenic of the three strains, had the largest increase in NFKBIA expression in BAL fluid. Ondangwa also had large increases in expression, but Shawawa did not. This correlates with the relative pathogenicity of each MmmSC strain. However, this was not the case in bronchial biopsy or nasal cytology samples, in which NFKBIA expression was inconsistent and variable. The BAL results suggest that NFKBIA may inhibit transcription of genes involved in disease pathogenesis, but determining the identity of these genes will require further investigation.

Interleukin 18 is best known as an inducer of proliferation of and IFNγ production by $T_H1$ cells and NK cells. It also promotes differentiation of T cells into the $T_H1$ phenotype. Mycoplasmal infections have been shown to induce IL18 production without IFNγ production in both humans and swine[192-195]. MmmSC has been demonstrated to depress IFNγ secretion,[67] although assessment of IL18 was not

conducted in that experiment. IFNγ production has been linked to survival in animal models of mycoplasmal infection including CBPP[65,97]. The presence of increased amounts of IL18 mRNA in Ondangwa- and Shawawa-infected cattle BAL and bronchial biopsy samples and the lesser amounts of IL18 in Gladysdale-infected samples might argue toward support of the IFNγ-dependent recovery hypothesis, with more pathogenic strains of MmmSC depressing the IL18 and IFNγ responses. This would not take into account the potential for IL18 expression without IFNγ production as has been described. This possibility should be considered in MmmSC infection, since our microarray data fail to show increased expression of IFNγ in the face of IL18 expression in Gladysdale-infected animals.

Calmodulin 2 is one of a family of calcium-binding proteins of which nearly 20 members have been found. Calmodulins are cytosolic proteins that bind to other proteins as activators. Their functions include roles in growth and the cell cycle as well as signal transduction and neurotransmitter synthesis and release. Calmodulin has been minimally investigated in the context of mycoplasmal infections, with most evidence suggesting that infection with mycoplasma induces release of calmodulin from the affected cell[196,197]. Investigation of intracellular calmodulin in a swine mycoplasma model failed to find any difference between infected and control tissues[198], so the increased extracellular calmodulin found in the other studies may reflect only cellular damage rather than actual change in calmodulin production. There was no discernable difference in CALM2 expression between our three strains when examining BAL or nasal cytology samples. In bronchial biopsy samples, strain Ondangwa demonstrated a moderate but

persistent downregulation, but the significance of this in disease pathogenesis is unknown.

Ferritin heavy polypeptide 1 (FTH1) encodes the heavy subunit of ferritin, the major intracellular iron storage protein in prokaryotes and eukaryotes. The best-characterized role of ferritin is cellular storage of iron in a soluble and nontoxic state. Sequestration of iron is a well-recognized host innate immune system strategy during microbial infection, although it is usually considered most significant during infection with intracellular organisms. Lactoferrin is considered to have the primary role in iron sequestration, but there is evidence that ferritin may also play a role[199,200]. No investigations have been done toward the role of host iron sequestration in resolution of mycoplasmal infection. One array-based bacteriologic study demonstrated transcriptional changes in mycoplasma grown under iron-restricted conditions[201], indicating that mycoplasma may be susceptible to iron sequestration strategies by the host animal. Comparison of our three MmmSC strains showed little correlation of FTH1 expression with disease pathogenicity. The only consistent alteration in expression was moderate upregulation of FTH1 in Ondangwa-infected BAL samples. Whether the increase in FTH1 expression in animals infected with strain Ondangwa has any bearing on the severity of lesions or disease resolution is unknown.

The cytokine interleukin 8 is a macrophage-secreted CXC chemokine best known as a neutrophil chemoattractant, but it has secondary roles in T cell and basophil chemotaxis. The single study investigating IL8 in mycoplasmal infection identified increased levels of the cytokine in swine tracheal explants[161], but did not propose a

mechanism. An experiment using a mycoplasmal lipid-associated membrane protein also stimulated production of IL8 by human monocytes and identified the mitogen active protein kinase (MAPK) cascade as a downstream pathway[202]. Our data would indicate a potent role for IL8 in host response to CBPP, as all strains demonstrated dramatic increases in IL8 gene expression in BAL fluid. Both Gladysdale and Ondangwa exhibited over 100-fold increases in IL8 expression over baseline but showed differential timing of maximal expression. However, given that all strains showed upregulation, this cytokine seems unlikely to be a primary determinant of differential pathogenicity between strains based on BAL samples. Bronchial biopsy samples showing increased IL8 expression of Gladysdale-infected animals over Ondangwa- or Shawawa-infected animals may imply greater neutrophil-induced oxidative damage in bronchial epithelium.

Mitogen activated protein kinase 1 (MAPK1), also known as extracellular signal-regulated kinase (ERK), is involved in a number of cellular processes including differentiation, proliferation and transcriptional regulation. Activation of MAPK1 requires its phosphorylation by upstream kinases. Following activation, MAPK1 translocates to the nucleus of the cell and phosphorylates nuclear targets. As mentioned in the previous paragraph, MAPK1 is part of a signaling cascade resulting in the transcription of cytokines including IL8. A previous study by the same group identified MAPK1/ERK as necessary in the signal transduction for transcription of IL1B and TNFα, but not IL6, following exposure to mycoplasmal lipoproteins[203]. Among the three MmmSC strains evaluated in our study, the Gladysdale isolate showed the lowest

MAPK1 expression but the highest IL8 and IL1B expression in BAL samples. Animals infected with strains Ondangwa and Shawawa showed greater BAL transcription of MAPK1 yet lower expression of the proinflammatory cytokines whose transcription is controlled by MAPK1. This suggests the involvement of other downstream regulatory mechanisms that may interrupt the signal transduction cascade prior to transcription. Candidates for this include the kinases that influence the phosphorylation state of MAPK1 such as DUSP14 or PTPN7. Biopsy samples showed higher expression of MAPK1 in Ondangwa-infected animals and nasal cytology samples did not demonstrate a consistent expression pattern. The host MAPK1 response to infection with certain other bacteria is believed to be calmodulin-dependent;[204,205] but our data do not appear to link calmodulin expression with MAPK1 expression.

Interleukin 1B is another macrophage-secreted cytokine whose roles include pyrogenesis, stimulation of acute-phase protein synthesis, increasing expression of adhesion molecules, platelet and fibroblast proliferation, T and B cell activation and induction of additional proinflammatory cytokines including IL6 and IL8. IL1B is a downstream product of the MAPK/ERK signaling cascade. IL1B expression has been shown to be dependent on calmodulin in an LPS-induced model[206], but the calmodulin-dependent IL1B pathway has not been confirmed for mycoplasmas. Multiple mycoplasmas have been shown to induce IL1B production, including *M. bovis, M. synoviae, M. pneumoniae, M. hyopneumoniae* and *M. pulmonis*[207-211]. MmmSC's ability to induce IL1B production has not been evaluated, and only one study has investigated IL1B secretion in any mycoides cluster organism[212]. Our study demonstrates the

induction of IL1B gene expression in BAL fluid of at least fourfold over baseline by all three strains of MmmSC in infected cattle. The magnitude of strain differences in BAL IL1B expression reflected the relative severity of disease induced by each strain, with Gladysdale>Ondangwa>Shawawa. Slight increases in expression were seen in bronchial biopsy samples but only reached fourfold over baseline in animals infected with strain Gladysdale. Gladysdale animals also showed a brief spike in IL1B expression in nasal cytology samples on Day 1 only. Our data suggest that IL1B likely has a role in the pathogenesis of CBPP.

The protein encoded by the gene signal transducer and activator of transcription 3 (STAT3) is a member of a group of proteins that is phosphorylated by receptor-associated kinases in response to cytokines and growth factors. Following phosphorylation, STAT proteins form homo- or heterodimers that function as transcriptional activators after translocation to the cell nucleus[213]. Some of the cytokines and growth factors known to induce phosphorylation of STAT3 are interferons, epidermal growth factor, IL5, IL6, hepatocyte growth factor, leukemia inhibitory factor and bone morphogenetic protein 2[214]. This protein mediates gene expression for a number of genes, depending on the specific stimulus, and thus plays a key role in many cellular processes including apoptosis and cell cycle. While no studies have investigated the role of STAT3 in mycoplasmal infection, investigations involving other bacteria have assigned STAT3 roles including that of a mediator of T cell activation[215] and neutrophil recruitment[216]. One model using *Salmonella* porins demonstrated a dependence of STAT3 on the MAPK pathway instead of its well-known dependence on

Janus kinase (JAK) phosphorylation for activation[217]. In our experiment, animals infected with strain Ondangwa demonstrated a consistent increase in STAT expression in BAL fluid while Shawawa-infected animals showed a single high peak on Day 8. Bronchoalveolar lavage STAT3 expression in Ondangwa-infected animals roughly paralleled MAPK1 expression, but did not do so in other sample types. No parallels could be seen between STAT3 expression and IL6 expression in any sample type. Investigation of the STAT pathway may identify dependent genes in mycoplasmal infection.

Comparison of surviving and nonsurviving animals infected with strain Gladysdale identified some intriguing avenues for exploration. While bronchoalveolar lavage expression of IL6 did not differentiate between survivors and nonsurvivors, evaluation of IL6 mRNA in blood samples demonstrated increased expression in nonsurviving cattle. The same comparison in nasal brushings showed the opposite pattern, with surviving cattle exhibiting greater expression of IL6. This is somewhat in conflict with the murine study mentioned previously, in which IL6 was proposed as a mediator of lung injury[187]; that investigation would imply that nonsurvivors should have greater expression of IL6 in the lung. That study, however, evaluated pulmonary IL6 transcription factors rather than BAL fluid cytokine, and expression may well differ between lung tissue and pulmonary macrophages. In addition, quantity of a transcription factor does not necessarily correlate with quantity of its cytokine.

Evaluation of NFKBIA showed that nonsurvivors more than doubled expression in BAL fluid while survivors had a fourfold increase in nasal epithelial cells. The

situation in BAL fluid correlates with hypotheses of NFKBIA's role in the pathogenesis of other infectious disease[191]. NFKBIA inhibits NFKB-mediated cytokine transcription, and absence of specific cytokines may negatively influence disease outcome. If NFKBIA is inhibiting cytokine expression in our model it would appear not to be doing so for either IL6 or IL8, which showed increased expression in both groups of animals. Interleukin 18, however, does show decreased expression in BAL fluid of nonsurvivors; perhaps it might be proposed as a candidate for NFKBIA-mediated downregulation. Bronchoalveolar lavage IL18 expression in nonsurvivors versus survivors was in agreement with other reports suggesting that IL18 production and subsequent IFNγ secretion enhance survival in mycoplasmal infections[65,194]. No other sample types exhibited this pattern.

Calmodulin 2 showed greater downregulation in nonsurvivors than survivors in BAL fluid, while alterations of expression in other sample types were not sufficiently large to draw conclusions. Calmodulin's role as a transcriptional activator implies that decreases in CALM2 expression may have far-reaching consequences on downstream activation of other genes. However, the apparent decrease in calmodulin expression in our nonsurvivor group does not appear to have affected IL8 expression, a relationship found in cells treated with *Mycobacterium* products[204] and *Clostridium* toxins[218]. The role of CALM2 in MmmSC infection cannot be hypothesized from this data.

Ferritin heavy chain 1 showed differential expression only in nasal brushing samples, in which survivors had a three- to fivefold increase in expression over nonsurvivors. This could reflect successful sequestration of iron by host cells leading to

elimination of the mycoplasma, but if that were the case the difference should also be observable in BAL or bronchial biopsy samples closer to the site of maximum mycoplasma presence. Further investigation is needed to elucidate the involvement of FTH1 in CBPP.

IL8 expression, while dramatically increased in both groups' BAL samples, was greater in nonsurvivors. This was reversed in biopsy and cytology samples, but only on Day 1. The situation in BAL samples would appear to have a rational explanation in that the increased recruitment of neutrophils to the lung would result in oxidative damage that could negatively impact survival. The IL8 expression in biopsy samples, however, would argue against such a hypothesis since the animals with greater expression survived. The true situation may be a matter of both degree and time; bronchial biopsy samples exhibited both a lower total fold change and a shorter period of highly increased expression than did BAL. Infected animals could potentially have a better chance of resolving less severe oxidative damage of short duration than profound oxidative damage lasting more than a week.

Such comparisons as are presented here are unlikely to be useful in prognosticating survival of individual cattle, since standard response to a CBPP outbreak in most countries is immediate slaughter of any affected animals. Additionally, true recovery from CBPP cannot be inferred since even the survivor group still had lesions at necropsy. However, this data may provide some indication of potential mediators of virulence, which in turn could provide clues toward intervention or vaccine development as well as disease pathogenesis. Any conclusions drawn from this comparison must

keep in mind that the sample size in this study was very small and verification with larger groups of animals will be necessary to validate this data.

In summary, specific host genes were identified that could provide insight into the mechanisms of differential strain virulence in MmmSC through understanding their effects on the host animal. Additionally, comparison of gene expression between survivors and nonsurvivors of a highly pathogenic strain of MmmSC may also identify pathways involved in disease pathogenesis.

# CHAPTER V

# CONCLUSIONS

*Mycoplasma mycoides mycoides* small colony is the causative agent of Contagious Bovine Pleuropneumonia, a devastating cause of cattle pneumonia affecting large parts of Africa, Asia and the Middle East. MmmSC is one of a cluster of closely-related mycoides organisms that are impossible to differentiate serologically. MmmSC is the only bacterial pathogen on the OIE list A, and is of interest in the United States because of its potential for deliberate introduction as an agroterrorism agent. The objective of this study was to establish a model of bovine infection with three strains of MmmSC not previously investigated, and to determine the bovine host response to infection with MmmSC through characterization of the transcriptome throughout infection. The hypothesis was that MmmSC infection causes alterations in the host transcriptome that may provide insight into how the mycoplasma establishes and maintains infection through interaction with the host.

In order to test our hypothesis, we developed models of infection using endobronchial inoculation of one of three African/Australian MmmSC strains of varying pathogenicity. Equal bacterial numbers of each strain were used. Animals were followed through infection, recovery, euthanasia and necropsy, and a timeline of clinical disease expression was described for each MmmSC strain. Gross and histologic evaluation of selected tissues completed the model.

The next goal was to characterize the temporal transcriptional profile of the bovine host during infection with the virulent MmmSC strain Gladysdale. Very little has been investigated with regard to the interaction of MmmSC with the host animal, and no gene expression studies have previously been conducted with this organism or its host. Toward this end, weekly samples were obtained of bronchoalveolar lavage fluid, bronchial epithelium, nasal epithelium and blood from infected cattle. Microarray analysis of RNA revealed a greatly upregulated transcriptional profile shortly after infection, with a gradual return of expression toward baseline levels over time. Cellular pathways intimately involved with establishment and maintenance of disease were identified using *in silico* analysis, and numerous mechanistic genes were identified in each tissue type analyzed. Many of the genes identified have not previously been associated with disease pathogenesis and further experimentation is required to elucidate their role in infection.

The next goal of this project was to directly compare gene expression of selected host genes between animals infected with each of the three MmmSC strains. The host response to infection can itself cause a portion of the pathology of the disease, as many components of the immune system are potent inflammatory mediators. Several genes, particularly cytokines and transcription factors, were found to be differentially expressed between the strains, indicating that they may play a role in disease pathogenesis. Genes that appeared to influence disease severity included IL6, IL8, IL1B, IL18 and NFKBIA. Not all genes investigated proved to have differential expression between the strains; genes that did not demonstrate a link with differential disease severity were CALM2,

FTH1, MAPK1 and STAT3. This does not eliminate these genes from a role in disease pathogenesis; they may be vitally important in the response to infection despite not being determinants of strain virulence. Additional investigation, such as that using RNA interference, is needed to more definitively identify the precise role of each of the potentially outcome-influencing genes.

The final goal of this project was to investigate gene expression between survivors and nonsurvivors of infection with strain Gladysdale. Differences in host gene expression between animals recovering from infection and those succumbing to infection with the same strain may highlight host genes important in the response to infection. For example, a gene that is differentially expressed between survivors and nonsurvivors may represent a gene whose product is vital in resolving infection. Alternatively, it may represent a gene whose product, when overexpressed, is involved in self-damage and worsening of disease. All genes evaluated demonstrated differential expression between survivors and nonsurvivors in at least one sample type; the nature of each gene's influence on survival remains to be determined.

These discoveries may be extended through the use of additional techniques including RNA interference, laser capture microdissection, targeted lymphatic tissue sampling or lymphatic cannulation to further define pathways and genes expressed by the bovine host. In addition, investigation of the very early host response (prior to 24 hours) may yield insight into the mechanisms of establishment of infection. Study of the causative organism will also provide valuable data. Although mycoplasmas are highly resistant to genetic manipulation, their small genome would allow use of array-based

analysis techniques with relative ease. Simultaneous exploration of host and bacterial gene expression *in vivo* would be particularly valuable, as identification of bacterial virulence factors in mycoplasmas has been elusive. Synthesis of this information will improve understanding of the bovine:MmmSC interaction, potentially allowing the development of sensitive and specific cow-side diagnostics and effective, safe vaccines toward an ultimate goal of control or even eradication of this pathogen.

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# APPENDIX A

## HIGHLY-ACTIVATED GENE ONTOLOGY GENES AND PROCESSES IN BRONCHOALVEOLAR LAVAGE SAMPLES

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Appendix A

# APPENDIX B

## GENE ONTOLOGY MECHANISTIC GENE LIST FOR BRONCHOALVEOLAR LAVAGE SAMPLES

This Excel file accompanies this .pdf file and is available for downloading as filename

Appendix B

# APPENDIX C

## HIGHLY-RANKED KEGG PATHWAYS AND GENES FOR BRONCHOALVEOLAR LAVAGE SAMPLES

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Appendix C

# APPENDIX D

## HIGHLY-ACTIVATED GENE ONTOLOGY GENES AND PROCESSES IN BRONCHIAL BIOPSY SAMPLES

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Appendix D

# APPENDIX E

## GENE ONTOLOGY MECHANISTIC GENE LIST FOR BRONCHIAL BIOPSY SAMPLES

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Appendix E

# APPENDIX F

## HIGHLY-RANKED KEGG PATHWAYS AND GENES FOR BRONCHIAL BIOPSY SAMPLES

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Appendix F

# APPENDIX G

## HIGHLY-ACTIVATED GENE ONTOLOGY GENES AND PROCESSES IN NASAL CYTOLOGY SAMPLES

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Appendix G

# APPENDIX H

## GENE ONTOLOGY MECHANISTIC GENE LIST FOR NASAL CYTOLOGY SAMPLES

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Appendix H

# APPENDIX I

## HIGHLY-RANKED KEGG PATHWAYS AND GENES FOR NASAL CYTOLOGY SAMPLES

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Appendix I

# APPENDIX J

## HIGHLY-ACTIVATED GENE ONTOLOGY GENES AND PROCESSES IN BLOOD SAMPLES

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Appendix J

# APPENDIX K

## GENE ONTOLOGY MECHANISTIC GENE LIST FOR BLOOD SAMPLES

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Appendix K

# APPENDIX L

## HIGHLY-RANKED KEGG PATHWAYS AND GENES FOR BLOOD SAMPLES

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Appendix L

# VITA


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